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The Carbohydrate Metabolism of Leukocytes: *A Review**

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INTRODUCTION

Because leukocytes arise from tissues of unusual growth potential and because of their predilection for leukemic involvement, it has been assumed that they might have metabolic traits in common with embryonic or tumor tissue. This question has attracted students for half a century. The wide diversity in motivation for these studies, ranging from the purely biochemical to the purely clinical, is reflected in a highly contradictory and dissentient literature. In the hope of delineating the present status of this work, it is proposed to review here the various past contributions on leukocyte carbohydrate metabolism with emphasis on studies of circulating blood leukocytes in health and leukemia, together with a critique of the methods used and the materials studied by past workers.

Investigations into the nature and etiology of leukemia have taken many directions, as pointed out recently by Furth (21). He states that most early theories were based upon clinical and morphological observations. The finding of a filtrable agent causing fowl "leukemia" and the subsequent studies on viruses, biological properties of leukemic cells, chemical carcinogenesis, endocrine and genetic influences, effects of radiation, and leukocyte elimination mechanisms have produced a welter of complex data and intriguing hypotheses. Furth states that present knowledge seems to indicate "the essential change in leukemia resides in the leukemic cell and consists of an acquired inability of immature leukocytes to respond to forces normally regulating their proliferation and maturation."

Leukemia is an abnormality that eventually and perhaps inevitably involves more than a single blood constituent, the white cells (13). Although leukocyte proliferation is the most obvious clinical finding, the entity of leukemia apparently includes disturbances in the erythrocytes, thrombocytes, and total body metabolism. It is not improbable that etiological information may be found in systems other than the leukocytes or their precursors. Conceivably, the disorder is one of growth control, a disturbance describable only in quantitative and kinetic terms and one which leaves little or no imprint on the cells themselves.

Even though it has not been established whether the metabolic changes discernible in leukemic cells themselves are a cause, accompaniment, or consequence of the disease process, much work has been done on the biochemistry of leukocytes. The advent of satisfactory methods for separating leukocytes from whole blood (11, 33, 50, 55) has made possible quantitative biochemical measurements with the methods of the tissue physiologist. The contributions of this type have been reviewed (52). In general, it appears that striking differences are to be found in the leukocyte content of various substances in health and leukemia. For example, the leukocytes of chronic myelocytic leukemia are high in histamine (54) and low in alkaline phosphatase (53). Those of neutrophilic leukocytosis show the opposite picture—low histamine and high alkaline phosphatase. The leukocytes of polycythemia vera which has passed into the so-called "leukemic" stage, with elevated leukocyte counts and cell immaturity, show high alkaline phosphatase values, the converse of the picture in typical chronic myelocytic leukemia (6). In addition, the leukocytes of polycythemia have high glycogen

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levels (60). Here, biochemistry provides a basis for classification where morphology has ceased to be adequate.

Circulating leukocytes are uniquely well suited for metabolic study under simulated *in vivo* conditions. With the exception of spermatozoa and certain unusual tumors such as the Ehrlich ascites tumor, leukocytes and their precursors are probably the only accessible free-floating, nucleated, mammalian cells. Certainly they are the most easily obtainable human cells. They can be accurately enumerated in the hemocytometer, a notable technical advantage, and can be precisely examined by a variety of well known cytomorphological technics.

Because they can be studied in the human patient, the leukocytes provide one of the few meeting grounds for cellular biochemistry and clinical investigation. These cells have the advantage of day-to-day availability in individuals undergoing changes in clinical and therapeutic status, thus permitting controlled biochemical studies with a dimension in time. In addition to leukocytosis and leukemia, leukocytes apparently participate in certain systemic metabolic disorders such as glycogen storage disease (59). Increased deposition of glycogen in leukocytes is reported to follow ACTH injection in rats (45). One wonders to what further extent leukocytes may participate in systemic metabolic or endocrine processes.

Circulating leukocytes lack many of the disadvantages inherent in solid tumor material, among which are the difficulties in obtaining homogeneous, stroma-free tumor cell preparations, the impossibility of accurately counting the cells of a solid tissue and, hence, the difficulty in expressing data, the uncertainty as to vascular and nutritional uniformity throughout the tumor mass, the possibility of immunological reactions between transplanted tumor and implanted host and the question of appropriateness of choice of normal or control homologous tissue. Morphological identification of cell type of origin may be extremely difficult in anaplastic tumors. This last difficulty is, of course, shared by leukocytes, concerning questions which are constantly arising as to the role of cell type and cell immaturity in determining the biochemical picture. This problem can sometimes be circumvented—e.g., by the study of patients in remission or under varying states of the differential formula, by inference from histochemical data, and by manipulations of the cell suspension which take advantage of the differences in specific gravity and sedimentation behavior of various cell types. The problem which plagues the biochemist studying all tissues, including leukocytes, still remains. It is the problem of somehow connecting, if not causally then by some other consistent correlation, the events of metabolism with the patterns of biological behavior. Here, leukocytes present no advantages, and one always risks the interpretive perils of transitional states which are neither black nor white, hazards which are deeply rooted in the issues of semantics.

REVIEW OF THE LITERATURE

The difficulties which arise in attempting to compare the results of different workers are chiefly three: (*a*) The materials studied differ widely in species of origin, anatomical source, cell type, stage of maturity, and mode of preparation. The literature reviewed will largely include studies on human and animal *peripheral blood leukocytes* from normal and leukemic individuals and *exudate leukocytes* obtained variously from human and animal empyema cavities, peritoneal exudates, sterile blisters, etc., although mention is made of pertinent collateral studies on solid lymphoid tissue, bone marrow, and "leukemic tumors." Where possible, technical details are given. (*b*) Differing analytical methods and incubation media have been used. It is concluded here that results are profoundly affected by the composition of the medium. (*c*) Metabolic data have been referred variously to dry weight, cell numbers, and to cell nitrogen. The authors consider cell numbers to be the most plausible reference basis in leukocyte studies and in reviewing past work we will, where possible, convert data into millimoles per 10^{10} cells per hour. Where the "Q" notation is used (indicating activity/mg dry weight/hour), an empirical factor of 1,960 is used to convert dry weight to 10^{10} cells.[1] ($Q_{O_2} \times 1{,}960 = \mu l/10^{10}$ cells/hr; 22.4 ml. O_2, 22.4 ml. CO_2, 90 mg. lactic acid and 180 mg. glucose are equivalent to 1 mM.) This procedure is intended only to facilitate rough comparisons.

Glycolysis and respiration.—It was early known that sugar disappeared from blood on standing, and, in the more primitive days of enzymology and analytical chemistry, this was variously attributed to blood "decomposition," and protein, sugar, or amino acid breakdown. In 1911, Slosse (48) noted that blood contained lactic acid which increased on standing. The source of the lactic acid was shown to be glucose, not protein or amino acids. The following year, Levene and Meyer (31), using quantitative methods, studied the conversion of glucose to lactic acid in leukocytes obtained from the pleural cavity of dogs after prior injection of tur-

[1] Obtained from the studies of Bird, Clements, and Becker (9) on nonleukemic cells and data of our own which are substantially in agreement.

pentine, and concluded that all glucose utilized could be accounted for by the lactic acid produced. Glucose was measured reductimetrically and lactic acid gravimetrically. Phosphate was found essential for activity. Because of the contemporary argument whether lactic acid was derived from carbohydrate or protein, this work had general significance. Hematologic data on the leukocyte material are not given, but it is known (14) that such exudate procedures yield a population of cells consisting almost exclusively of polymorphonuclear neutrophils. Levene and Meyer (32), extending their observations, found that fructose, mannose, and galactose, but not pentoses, were transformed into lactic acid by leukocytes.

Rona and Arnheim (46) compared the glycolytic activity of white and red cells and found the latter greater, no doubt because of the larger number of red cells present in blood since MacLean and Weir (36) in 1915 showed, by comparing bloods with varying leukocyte counts, that the ratio of glycolytic activity (per cell) of white to red cells varied from 200:1 to 1000:1. In five experiments on a plasma suspension rich in normal leukocytes and a glucose concentration of 0.0015 M, glucose utilization averaged 3.50 mM/10^{10} cells/hr, as measured by a reducing method.

The first study of the *respiratory activity* of leukocytes was that of Grafe (24) in 1911. He attempted to explain the marked increase in the total O_2 consumption of the body in leukemia with data on the gas exchange of the blood leukocytes. The cells were obtained by centrifuging whole blood, removing the plasma, and resuspending red and white cells in a salt solution containing saponin. Using a Haldane-Barcroft apparatus, he found O_2 consumption rates of 0.037–0.082 ml/10^5 cells/hr (0.017–0.037 mM/10^{10} cells/hr) in a group of myelocytic and lymphocytic leukemics. Glycolysis was not measured. The gas exchange of the polymorphonuclear leukocyte was considerably higher than that of the mononuclear.

The semi-quantitative studies of Fukushima (20) in 1922 and Löhner (34) in 1926 on glucose utilization by leukocytes obtained from rabbit peritoneal exudate, horse blood, and human empyema pus showed that sugar breakdown continues *in vitro* for about 72 hours and that the washed leukocytes themselves contain little sugar or reducing substance.

Are leukemic leukocytes "embryonic" or "malignant" cells?—During this period, the classic studies of Warburg (63) made their appearance. It was observed that slices of cancer tissue possessed a greater capacity for aerobic and anaerobic transformation of glucose into lactic acid than did normal tissue, tumor tissue exhibiting a deficient Pasteur effect. High anaerobic glycolysis was also found in embryonal tissue, suggesting a connection between anaerobic glycolysis and rapid growth. In the embryo, however, glycolysis was largely abolished in oxygen. It was concluded that neoplastic transformation involved fundamental changes in energy-supplying metabolic reactions. A vast number of confirmatory reports strongly implied that here one had criteria for the state of malignancy: high aerobic and anaerobic glycolysis, a low relative Pasteur effect, and a moderately high respiratory rate. The eventual discovery of exceptions, of normal tissues possessing these characteristics such as retina, kidney medulla, and jejunal mucosa, made it clear that the *specificity* of the tumor metabolism pattern was illusory. An attempt was made to attribute the so-called "cancer pattern" when found in normal tissues to injury or damage in handling. Thus began a long cycle of studies which sought to label the various tissues as normal, injured, cancer-like, or embryo-like.

Bakker (2) believed that leukocytes obtained from rabbit peritoneal exudate (presumably polymorphonuclear neutrophils) behaved like cancer cells, because he observed a moderate oxidative level, a high anaerobic and a fairly high aerobic glycolysis. Using a saline suspension of intact leukocytes, he found in typical experiments that the addition of glucose (0.1 per cent) increased the 1-hour O_2 consumption by 72 per cent and that the ratio of aerobic glycolysis to respiration was approximately 25:1.

Daland and Isaacs (12) measured the O_2 consumption of human whole blood in six normal individuals and ten patients with leukemia. Activity in the normal cells was too low to measure and that in the cells of myelocytic leukemia averaged 765 μl/100 ml of blood/hr. It seemed to the authors that the results could be best correlated with the number of mature polymorphonuclear leukocytes present, that the Q_{O_2} of the immature cells was probably low, and, therefore, they resembled tumor cells more than embryonic or young tissue.

This conclusion was disputed by Fujita (19), who observed that leukocyte metabolism resembled that of normal embryonic, not malignant, tissue. He incubated a centrifuged fraction of rat blood containing 60 vol. per cent of intact leukocytes in a citrate-plasma medium and found a Q_{O_2} of 9.2 (0.80 mM/10^{10} cells/hr) a $Q_M^{O_2}$ of 2.6 (0.56 mM/10^{10} cells/hr), and a $Q_M^{N_2}$ of 20.6 (1.80 mM/10^{10} cells/hr.), indicating a "replacement" of anaerobic glycolysis by respiration.[2] The ratio of

[2] $Q_M^{N_2}$ and $Q_M^{O_2}$ signify mg of lactic acid produced/mg dry weight/hour in N_2 and O_2, respectively.

$Q_{M}^{O_2}$ to Q_{O_2} in terms of glucose equivalents was 0.85 to 1. Only two experiments were quoted by the author, and technical details were not given. Presumably, the leukocyte population had the type distribution of average normal rat blood, although this is not made clear. The author stated without qualification that prolonged centrifugation and cooling to 0° C. significantly lowered the leukocyte respiratory rate.

A comparison of the metabolism of blood leukocytes (of geese) with exudate leukocytes (of the rabbit) was made by Fleischmann and Kubowitz (18), using Ringers-phosphate as the incubation medium. It was found that the aerobic glycolysis of exudate leukocytes ($Q_{M}^{O_2} = 14.0$) was higher than that of blood leukocytes ($Q_{M}^{O_2} = 1.8$). The high aerobic glycolysis rate in exudate was taken to mean that aerobic glycolysis in leukocytes is not proof of the malignant state but due instead to injury, since "leukocytes are dying more rapidly in exudate than in blood." No comment was made on the possible significance of species difference. The reported Q_{O_2} rates were around 4.5, 10 times higher than those reported by Bakker (2).

A succession of authors in the 1930's continued debating over the possibility of differences between normal and leukemic cell metabolism, on the relationship between cell maturity and cell metabolism and on the issue of whether leukemic leukocytes were indeed malignant, normal, embryonic, or injured. The controversy failed to be resolved. In 1930, Peschel (41) examined the lymphocytes of four cases of human lymphatic leukemia. The cells were isolated by centrifugation and were suspended in 0.016 M bicarbonate containing 160 mg. per cent glucose. The mean Q_{O_2} was 5.8, $Q_{M}^{O_2}$ zero, and $Q_{M}^{N_2}$ 11.0—seemingly a pure oxidative metabolism with no aerobic glycolysis. This was confirmed by Schlossmann (47), both authors concluding that the lymphocytes of lymphatic leukemia were young normal cells. The appearance of aerobic glycolysis in these cells was an "accompaniment of cell aging and death."

The studies of Bossa (10) on human leukocytes obtained by brief centrifugation showed a high aerobic glycolysis ($Q_{M}^{O_2} = 4.2$–37.3) in myelocytic leukemia but a very low aerobic glycolysis in leukemic lymphocytes. Bossa considered that these findings signified, not cancer metabolism, but the results of cell injury. Rin (44) was unable to find any evidence of aerobic glycolysis in normal and leukemic leukocytes and concluded that these cells were of the embryonic type.

The field was reviewed by Kempner (30) in 1939. He studied patients with myelocytic and lymphocytic leukemia and concluded that aerobic glycolysis occurring in mature leukocytes was a symptom of aging and dying. As Fleischmann (17) said, "The aging of a cell in the blood stream or its wandering out of the blood into an exudate results in an aerobic glycolysis."

In attempting to characterize leukemic cell metabolism, Victor and Potter (56) found in the lymph nodes of mice, with spontaneous and transmitted lymphocytic leukemia, consistent increases in both aerobic and anaerobic glycolysis over the values obtained with normal nodes. Hall and Furth (25) presented similar results. Their data showed little difference between the oxygen consumption of lymph nodes of leukemic mice and that of normal lymph nodes.

Effect of cell immaturity.—In 1929, Barron and Harrop (4) undertook to compare the metabolic activities of human granulocytes and nongranulocytes and to determine the possible significance of cell maturity and certain experimental conditions. The authors first found that the metabolism was greatly influenced by the *in vitro* conditions, respiration being adversely affected by overcrowding of cells in the experimental flask, the use of citrate as an anticoagulant, prolonged experiments, cooling, and centrifugation. These restrictions necessitated quick, mild centrifugation of fresh blood and immediate transfer of an aliquot of cells (red and white) into the respirometer, avoiding cooling. Glucose disappearance was determined by the Benedict method, and glycolysis was assumed equivalent to glucose disappearance. Data were expressed per 10^6 cells. It was concluded that there were no differences in the metabolism of mature and immature granulocytes or of granulocytes from cases of leukocytosis and myelocytic leukemia. The conclusions are perhaps open to question in that myelocytic leukemia and leukocytosis were lumped together as the "polynuclear group," and chronic lymphocytic leukemia and dog lymph lymphocytes as the "mononuclear group." The latter cells were stated to have a higher O_2 consumption than lymphocytes from lymphocytic leukemia, but this was speculatively attributed to crowding in the leukemic material. In five cases of myelocytic leukemia, the average O_2 consumption was 0.80 μl/10^6 cells/hr (0.35 mM/10^{10} cells/hr), and the glucose utilization 0.0038 mg/10^6 cells/hr (0.20 mM/10^{10} cells/hr). The data suggested that the aerobic glycolytic activity in "polynuclear" cells was 5 times greater than that in the "mononuclear" cell.

Glover, Daland, and Schmitz (22) believed that the metabolism of leukemic whole blood was certainly related to the maturity of the leukocytes. In extremely blastic leukemia, the O_2 consumption

was lower and the aerobic glycolysis greater than in bloods with more mature cells. The authors determined the glycolytic rate by substracting from the total sugar utilized the amount oxidized, assuming that all oxygen utilized was used for the oxidation of sugar. The Q_{O_2} of normal human leukocytes was 7.1 (0.62 mM/10^{10} cells/hr) and that of chronic myelocytic leukemic cells 2.5 (0.23 mM/10^{10} cells/hr). Lymphocytic leukemic leukocytes varied from 2.4 to 5.8 (0.21 to 0.51 mM/10^{10} cells/hr).

The studies of Soffer and Wintrobe (49) failed to confirm Daland and Isaacs, and Glover, Daland, and Schmitz. These workers, studying whole bloods containing varying numbers of cells, concluded that the respiratory and glycolytic rates of leukemic cells were slightly higher than the normal although the differences were small. Furthermore, they could make no correlations with the level of maturity. They found the metabolism of granulocytes to resemble that of malignant tissues and lymphocytes that of normal adult tissue.

Ponder and MacLeod (42) attempted to evaluate the effects on metabolism of cell immaturity by producing rabbit peritoneal exudates in rapid succession, so that the cells became younger and younger due to bone marrow stimulation. The O_2 consumption of the first exudate was 0.18 mM/10^{10} cells/hr and that of the fourth exudate containing many immature forms was higher by 50 per cent. An increase of 400 per cent could be obtained by repeated exudates in rapid succession.

Keibl and Spitzy (27) recently reported studies on a group of human normal and leukemic leukocytes. Cells were isolated by erythrocyte sedimentation, and it is stated that centrifugation was not harmful. The authors compared respiration, and aerobic and anaerobic glycolysis in a group of selected cases showing graded variations in the differential formula. From these studies, differences are reported, for example, between cases of lymphocytic leukemia with 80 per cent lymphocytes and 20 per cent neutrophils and cases with 90 per cent lymphocytes and 10 per cent neutrophils, the latter reportedly showing significantly lower aerobic glycolytic rates. The authors conclude that myeloid cell respiration, both normal and leukemic, increases with increasing maturity. Unfortunately, there is no statement of the standard error encountered in making comparisons such as the above. The authors present only the mean results of their groups, and no mention is made of the large probable error in the differential counts which were determined "by the usual clinical methods."

Effect of added serum and cell crowding.—Workers have varied in their choices of suspending media for leukocyte preparations, some using serum and others different saline solutions. It is not always clear whether the serum medium was normal or leukemic serum, and seldom are the reasons given for the authors' choices. Victor and Potter (58) demonstrated that mouse leukemic serum is typically hypoglycemic. A number of reports have compared the activity of cells with and without added serum. Victor and Potter (57) showed that normal mouse lymphoid and leukemic cells have higher respiratory rates in normal serum than in Ringer's solution, while the anaerobic glycolytic rates are the same in both media. Typical experiments showed Q_{O_2}'s in leukemic cells of 6.1 (in serum) and 4.9 (in Ringer's); and, in normal cells, 5.5 (in serum) and 4.6 (in Ringer's). The effect of the suspending medium was also studied by MacLeod and Rhoads (37). Using suspensions of rabbit peritoneal exudate leukocytes, the authors observed in Ringer's-phosphate an average Q_{O_2} of 4.6, $Q_G^{O_2}$ of 17 and $Q_G^{N_2}$ of 25.[3] Serum, neutralized with HCl, increased the respiration to a mean Q_{O_2} of 7.0, an increase of 50 per cent. Similar findings were reported by Pages and Delaunay (40). The enhancement of respiration reported to occur on addition of serum probably reflects the addition of catalytic ions, coenzymes, and substrates, although Warren (64) failed to identify definitely the stimulatory factor of serum, his evidence indicating the possibility of a dicarboxylic acid.

It has been reported by some that leukocytes are peculiarly susceptible to crowding, i.e., respiratory rates do not increase linearly with an increase in cell numbers in the incubation flask. This problem was encountered by Soffer and Wintrobe (49) and, as mentioned above, by Barron and Harrop (4). Ponder and MacLeod (43) pointedly denied that centrifugation, cooling, or crowding interfered with leukocyte respiration, although the latter authors were dealing with rabbit peritoneal exudate leukocytes and the former with human material.

More recently, Hartman (26), using guinea pig exudate leukocytes in various concentrations, found that a disproportion between O_2 consumption per cell and cell concentration occurred only when the cells were suspended in serum but that proportionality existed when the leukocytes were washed and suspended in a physiological salt solution. In a typical experiment a serum suspension containing 0.315×10^8 leukocytes consumed 0.96 μl $O_2/10^6$ cells/hr (0.43 mM/10^{10} cells/hr) and one

[3] $Q_G^{O_2}$ and $Q_G^{N_2}$ signify μl. of CO_2 produced by action of formed acid on added bicarbonate/mg dry weight/hr in O_2 and N_2, respectively.

containing 5.04×10^8 cells consumed 0.47 μl $O_2/10^6$ cells/hr (0.21 mM/10^{10} cells/hr). No such difference was noted in the saline suspension. Beck and Valentine (7, 8), using isotonic KCl homogenates of human leukocytes, also found no crowding effect.

Effect of phagocytosis.—Baldridge and Gerard (3), working with dog leukocytes in serum, observed that the addition of *Sarcina lutea* caused an increase in O_2 consumption. The rise began immediately, lasted 10–15 minutes and ceased in 1½–2½ hours. No respiratory change accompanied ingestion of India ink. Ado (1) determined the effect of phagocytosis on the aerobic glycolysis and O_2 consumption of dog and guinea pig exudate leukocytes in Ringer's solution. The Q_{O_2} averaged 5.2 (0.45 mM/10^{10} cells/hr) regardless of the presence or absence of phagocytosis. The so-called Q_M^{KCN} (amount of lactic acid produced/mg dry weight of tissue/hr in a system made "anaerobic" with KCN) averaged 0.05 (1.10 mM/10^{10} cells/hr) in the absence of phagocytosis, 0.032 in the presence of starch granules, and 0.017 in the presence of staphylococci. The results obtained by Baldridge and Gerard were confirmed by Delaunay, Pages, and Maurin (15) and by Marinelarena (38).

Effect of therapy.—Keibl and Spitzy (28) studied the effects of radiation, urethan, nitrogen mustards, and colchicine on chronic myelocytic leukemia leukocyte metabolism and concluded that observed changes were attributable to therapy-induced alterations in the differential formula. The main change was increased respiration. Glycolysis was affected variably. Reports of various authors have shown that leukocyte respiration is decreased by potassium arsenite (65) and thiouracil (66) and is stimulated by menadione (51) and ascorbic acid (39).

Recent studies on isolated leukocytes.—Using newer technics of leukocyte isolation, measurements were made of O_2 consumption, glucose utilization, and lactic acid production in human nonleukemic and leukemic material by Bird, Clements, and Becker (9). Hematologic data are not given. Cells obtained by the bovine fibrinogen method (11) were incubated in a mixture of plasma, Ringer's solution, and added glucose (final concentration, 500 mg. per cent). Measurements were made of lactic acid formed, and glucose was determined by the reduction method of Benedict. Metabolic rates, first calculated per 10^6 cells/hr, were converted to a dry weight basis by using a universal factor determined separately on 10 specimens of normal cells. The use of this factor (1 mg dry wt. = 5.1×10^6 cells) for both nonleukemic and leukemic cells was justified by the statement that the nitrogen content of both normal and abnormal cells was similar. The data, here reconverted to a reference basis of 10^{10} cells (and to mM), indicated a mean O_2 consumption of 0.23 in nonleukemic leukocytes and 0.10 in both chronic myelocytic and lymphocytic leukemia. Lactic acid accounted for only 75 per cent of the total acid formed anaerobically by myelogenous leukocytes. The authors believed their data showed no significant difference between the metabolism of nonleukemic and myelocytic leukemic cells. A low glucose utilization and lactic acid formation was found in lymphocytic leukemia leukocytes and was considered statistically significant.

There have been several reports on the effect of varying substrate and coenzyme conditions in leukocyte material. Marinelarena (38) reported the respiratory and glycolytic activities of guinea pig exudate leukocytes with various substrates and inhibitors. Using intact, washed cells suspended in Ringer's solution, and conventional manometric technic, the author typically found an O_2 consumption around 0.46 μl/10^6 cells/hr and a glycolytic rate of 0.89 μl of $CO_2/10^6$ cells/hr. Homologous serum benefited respiration. Added glucose, hexose diphosphate, and fructose increased glycolysis, and succinate increased respiration. Glycolysis was decreased 76 per cent by fluoride, 0.1 M, and 88 per cent by iodoacetate, 0.001 M. KCN, 0.001 M, decreased respiration 67 per cent and malonate, 0.04 M, decreased it by 17 per cent.

McKinney, Rundles, and Martin (35) isolated intact normal human leukocytes by the dextran method and found, after incubation in a system containing plasma and Hank's solution, oxygen consumption of 0.28 mM/10^{10} cells/hr. This value was not increased by addition of citrate, α-ketoglutarate, succinate, fumarate, malate, oxalacetate, or glutamate in final concentrations of 0.01 M. Added cytochrome c (5×10^{-5} M) did not stimulate the respiration of the intact cells. However, when the cells were disrupted by grinding with alumina gel, stimulation of O_2 consumption did occur on addition of succinate, α-ketoglutarate, oxalacetate, and cytochrome c. The possible effect of adsorption of enzyme protein by alumina gel is not discussed.

Beck and Valentine (7, 8) studied the respiration and aerobic glycolysis of leukocytes isolated by the fibrinogen method (11) from normal and leukemic blood. Three clinically uniform groups of patients were assembled for study: normals; chronic myelocytic leukemics (average leukocyte count 100,000/c mm, with 96 per cent myeloid cells, including chiefly myelocytes, metamyelocytes and band forms); chronic lymphocytic leukemics

(average leukocyte count, 124,000/c mm, with 93 per cent lymphocytes.). Leukocytes were suspended in alkaline isotonic KCl, homogenized, and incubated in a buffered (pH 7.4), fortified system containing added DPN (0.0007 M), ATP (0.0011 M), cytochrome c (1.4×10^{-5} M), phosphate (0.01 M), Mg^{++} (0.005 M), and various substrates. Data were expressed per 10^{10} cells. Lactic acid was measured chemically and glucose utilization by a modification (5) of the glucose oxidase method of Keilin and Hartree (29), which, being more specific than reducing methods, gave demonstrably higher disappearance values. With glucose and hexose diphosphate (0.0053 M) as substrates, the following mean values were found for O_2 consumption, glucose utilization, and lactic acid production in mM/10^{10} cells/hr. (Standard deviations are shown):

	O_2 consumption	Glucose utilization	Lactic acid production
Normal	0.40±0.17	1.40±0.78	3.01±1.60
Chronic myelocytic leukemia	0.12±0.03	0.52±0.26	1.27±0.50
Chronic lymphocytic leukemia	0.11±0.01	0.31±0.08	0.48±0.19

The oxygen consumption, glucose utilization, and lactic acid production are significantly higher in normal than in leukemic homogenates. It is seen that, in this system, leukocytes have a predominantly aerobic glycolytic metabolism. In the presence of glucose and HDP, the ratio of aerobic glycolysis to respiration (in terms of glucose equivalents) is about 30 in normal and myelocytic leukemia and about fifteen in lymphocytic leukemia. In the absence of added substrate, the glycolysis to respiration ratio in normals is 3.5; myelocytic leukemia, 10.0; and lymphocytic leukemia, 1.6. Succinate stimulated oxygen uptake to a greater extent in normal than in leukemic material, and the omission or manipulation of added co-factors profoundly affected metabolic rates: both types of leukemic leukocytes had a greater cytochrome c requirement for maximal oxygen uptake and lymphocytic leukemic leukocytes had a greater DPN requirement for maximal lactic acid production than did normal leukocytes.

Metabolic pathways of leukocytes.—The many reported biochemical differences between normal and leukemic leukocytes suggest possible different pathways of metabolism in the two tissues. Studies, such as the authors', indicating differing responses to added substrates and coenzymes, support this possibility. The intermediary carbohydrate metabolism of leukocytes is being most carefully studied by Wagner and his associates. This group (61) studied the breakdown of glycogen in leukocytes and noted that the reaction was catalyzed by a phosphorylase. In leukocytes isolated by flotation technics from citrated horse blood, they demonstrated the formation of an intermediary reducing substance in the enzymatic breakdown of glycogen to lactic acid. This could be shown only by use of cell suspensions which had been dialyzed against distilled water. Without dialysis, the reducing intermediate did not accumulate, and the cells utilized inorganic phosphorus. The presence in the reaction mixture of adenosine-5-phosphate was essential for the formation of the reducing substance. Addition of NaF inhibited lactic acid formation without interfering with glycogen degradation and reducing substance formation. More recent studies (62) demonstrated inorganic phosphorus utilization in dialyzed cells. Organic phosphate intermediates, reducing and nonreducing, were isolated and identified as glucose 1-phosphate, glucose 6-phosphate, fructose 6-phosphate, phosphoglyceric acid, and fructose 1,6-diphosphate.

DISCUSSION AND SUMMARY

Both the older and the more recent literature would indicate that normal and leukemic myeloid cells are characterized by a substantial aerobic glycolysis. Conversely, lymphatic leukemic lymphocytes and blast forms have low aerobic glycolytic rates. Hence, it is improper to make conclusions regarding the neoplastic nature of leukemia on the basis of their "tumor metabolism."

The concept of "metabolism of injury" is also clearly hazardous, inasmuch as the claim that cells are injured is frequently based on the metabolic results obtained and on no other criteria. The absence of marked metabolic differences between intact cells and homogenates (7) and, in fact, the considerable difficulties in preparing leukocyte homogenates cast further doubt on the role of injury in producing a characteristic metabolic pattern.

It appears that normal myeloid leukocytes possess higher rates of respiration and glycolysis than do leukemic cells. It may be that leukocyte glycolysis suits these cells for existence in the comparatively anoxic environment of exudates. It has been shown (17) that phagocytosis persists in the presence of KCN as long as glucose is present. Fluoride and iodoacetate inhibit phagocytosis. Bone marrow is reported to have a similarly high aerobic glycolytic rate (16) and low oxygen consumption (23).

The problem of precisely what material to use as control in studying leukocyte abnormalities is admittedly far from solution. Ideally, leukemic lym-

phocytes should be compared to normal lymphocytes, blasts to blasts, and so on. As yet, such rigorous standards are all but unattainable, and one is forced to make inferences from more indirect approaches.

Leukocytes are extremely sensitive to the composition of the incubation medium. Recent results indicate certain qualitative and quantitative differences in the responses of normal and leukemic material to addition of various substrates and coenzymes. It is hoped that, in the light of more modern biochemical knowledge, further clarification may be brought to this problem.

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Fungi in Human Neoplastic Tissue*

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Recently, Diller and Fisher (1) reported the constant presence of fungi in many different types of animal and human neoplasms. They stated that "fungi imperfecti related to the Blastomycete group have been isolated from all types of tumors including human neoplasms. All types of human neoplasms thus far tested produced fungus growths when streaked in agar." The present microscopic and cultural investigation of human tumors was undertaken to evaluate these findings.

The pathogenic fungi have been shown to be strongly stained by the Hotchkiss-McManus technic (2, 3). This procedure is a most satisfactory way of detecting fungus cells in tissue.

METHODS

Sixty-three human tumors (49 malignant, fourteen benign) were surgically excised, wrapped in sterile gauze, and sent to the laboratory of surgical pathology. A small piece was cut out as aseptically as possible and transferred to a sterile Petri dish. The tumor fragment was then washed briefly in a solution containing 50 units each of streptomycin and penicillin. It was divided into two portions, each of which was inoculated into a Sabouraud's agar slant which was then incubated at room temperature. If no growth occurred within a month, the tube was discarded.

Microscopic sections were prepared from each tumor. These were stained according to both the hematoxylin-eosin and the Hotchkiss-McManus technics.

In addition, sections were cut from 192 paraffin blocks from our files of tumors. These were stained in a similar manner. They included 119 malignant tumors, 45 benign tumors and miscellaneous lesions, and 28 cases of sarcoidosis. The Hotchkiss-McManus-stained preparations were each viewed independently by two observers.

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RESULTS

Of the 49 malignant tumors cultured, eighteen (37 per cent) yielded no fungus growth. Thirty-one (63 per cent) yielded fungi, but, of these, fifteen were positive in only one of the two tubes.

The fungi recovered and their incidence in relation to the type of neoplasms examined are given in Table 1. Sixteen different fungi were recovered, and no one species occurred in more than 13 per cent of the positive cultures.

Of the fourteen benign lesions cultured, five (36 per cent) yielded no fungi; nine (64 per cent) yielded fungi, but, of these, five were positive in only one of the tubes.

The fungi recovered and their incidence are shown in Table 1. Nine different fungi were recovered, and no one species occurred in more than two (15 per cent) of the thirteen positive cultures.

The fourteen benign lesions cultured included the following: basal-cell tumors, leiomyomas of the uterus, fibroadenomas, nontoxic nodular goiters, thyroid adenomas, hemangiomas, mixed tumor of parotid, and cystadenomas of pancreas. One case of sarcoid that was cultured did not yield a fungus growth.

HOTCHKISS-MCMANUS STAINED SECTIONS

Of the 63 benign and malignant tumors that were both cultured and sectioned, none revealed the presence of fungi when stained with the Hotchkiss-McManus technic. An additional 119 malignant tumors, 45 benign lesions, and 28 biopsies from patients with sarcoidosis were stained. The malignant tumors included the following: 25 breast tumors, sixteen gastrointestinal tract tumors, twelve renal tumors, 24 skin tumors, six melanomas, eleven bronchial tumors, nine lymph nodes diagnosed as Hodgkin's disease, four sarcomas, six malignant tumors of the uterus and fallopian tubes, and six miscellaneous tumors. The 45 benign lesions included six thyroid tumors, ten skin lesions of psoriasis, eight lesions of molluscum contagiosum, eight lymph nodes diagnosed as reticulo-endothelial hyperplasia, four fibroadenomas, one lymph node diagnosed as chronic lymphadenitis, one neurilemoma, one mixed tu-

mor of the parotid, and six other miscellaneous benign lesions. Of a total of 255 biopsies examined histologically, only one revealed the presence of fungi. This was from a case of sarcoidosis in which a group of six oval fungus cells were encountered in a single section. Examination of additional sections from the same case, and even from the same block of tissue, failed to reveal fungi.

DISCUSSION

Approximately 36 per cent of all the lesions cultured were sterile. This is in direct contradiction to the work of Diller and Fisher (1) who found fungi by culture in all the human tumors studied. Again, the fungi isolated in the present study were those that are commonly regarded as laboratory contaminants. The contamination may have occurred in the operating room, in the laboratory, or the fungi may have actually been present in the tissues *in vivo*. Many of these specimens were obtained from organs where these fungi may normally be surface inhabitants, i.e., gastrointestinal tract, skin, etc. The source of contamination of this latter group of tissues is obvious. The fact that fungi could not be observed microscopically with a stain as definitive as the Hotchkiss-McManus stain, and yet could be isolated in culture so readily, suggests chance contamination during excision and handling of the tumors. Of course if only a few fungus cells were actually present in the tumor tissue, it is possible that they could have been missed histologically. In approximately one-half of the fungus-positive cases, only one of the two tubes inoculated from the same tumor was positive. Perhaps with more meticulous technic a greater percentage of sterile inoculations would have resulted.

Diller and Fisher (1) presented photographs of structures which they interpreted to be fungi parasitizing the nucleus. These structures scarcely resemble fungus cells. It is our impression that they are artifacts and that they are not uncommon in tumor tissue. No intranuclear fungus parasites are known at the present time.

Photographs are presented by these investigators (1) (Fig. 4, p. 96) in which elements designated as fungi are found in the cytoplasm. Their resemblance to fungus elements is superficial. They are not fungi in our estimation. Diller and Fisher did, however, show fungus filaments ramifying freely in Sarcoma 37 tissue. These tumors are notoriously subject to ulceration, and the presence of fungi in this situation is not at all surprising.

TABLE 1

ORGANISMS ISOLATED FROM MALIGNANT AND BENIGN TUMORS AND RELATION TO TYPE AND LOCATION OF NEOPLASM

	Malignant tumors									
	Carcinomas									
	Gastro-intestinal tract	Breast	Genito-urinary tract	Endo-crine	Respir-atory	Skin	Sarcomas	Lympho-mas	Totals	Benign tumors
Number of tissues examined	16	14	4	6	3	2	3	1	49	14
Positive*	11	10	2	3	2	1	2	0	31	9
Organisms										
Aspergillus†	2	1	1				1		5	1
Alternaria	1		2		1				4	2
Candida	4								4	
Rhodotorula		2				1			3	2
Mucor	2	1							3	
Neurospora	2								2	
Syncephalastrum				2					2	
Cryptococcus	1						1		2	1
Pullularia		1	1						2	1
Hormodendrum	1	1							2	1
True yeasts	1								1	
Cephalosporium		1							1	
Geotrichum		1							1	
Scopulariopsis		1							1	2
Paecilomyces				1					1	
Penicillium		1							1	2
Mycelia Sterila										1

* If either of the two culture tubes from a tissue specimen contained a fungus, the specimen was considered to be positive.

† If one or both of the two tubes inoculated from each specimen contained the same fungus, the organism was listed once. If each of the tubes contained a different fungus, each organism was listed separately.

SUMMARY AND CONCLUSIONS

1. Sixty-three benign and malignant tumors were cultured for fungi. Twenty-three specimens revealed no growth. From the other 40 specimens, sixteen different organisms were isolated. No one species of fungus was isolated in more than 15 per cent of the positive specimens.

2. Two hundred and fifty-five specimens, including malignant and benign tumors, 28 cases of

sarcoid and a number of miscellaneous non-neoplastic lesions, were examined by the Hotchkiss-McManus technic. No fungi were observed, except in one section from a case of sarcoidosis. Other sections from this same block of tissue failed to reveal fungi by staining.

3. It is concluded that the fungi isolated from human neoplasms were probably chance contaminants.

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Effects of Fractionated Doses of X-radiation on Normal and Tumor Tissue*

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Our investigations (5, 6) concerned with quantitative data on problems of growth, regeneration, and the differential response of normal tissue to changes induced by x-radiation in the testes of mice are now augmented by further data on testes, on germinal cells in the ovary, and on mouse tumors.

These studies confirm the belief that there is more than one kind of resting spermatogonia and that they differ in their resistance to x-radiation. The investigation also reveals that some germinal cells in the ovary have relatively the same order of radio-resistance as the corresponding cells in the testis. Some cells of the transplantable and spontaneous tumors are more resistant than the spermatogonia or oocytes in the gonads of the same species. Under the conditions of our experiment, it appears doubtful that fractionated doses of x-radiation produce resistance.

A review of the literature regarding the general effect of irradiation on the testis is included in a previous publication (5).

The differential response of the germinal elements in the testes of adult pure strain mice subjected to acute direct x-radiation follows a characteristic pattern as demonstrated by the quantitative random sampling method previously described (5, 6). It was shown that by plotting frequency of appearance of any of the germinal elements against time, for any of the doses used there was (*a*) a period of decrease, (*b*) a period where there were no alterations in the number of spermatogonia, and (*c*) recovery for the lower dose range. One purpose of this investigation was to ascertain what effect repeated doses would have on the spermatogonia of the testes when given at intervals determined by the time limits of any one of the critical periods enumerated above. The data obtained by using the sampling method provide a comparison of the effects of a single dose versus those of any equivalent fractionated dose.

A detailed discussion of over-all effects of irradiation on animals and an extensive review of the literature has been presented by Dr. T. C. Evans (3). It is indicated that fractionated doses of radiation are believed to have less effect than acute doses. This opinion is based on the assumption that some recovery may take place between exposures.

Melnick and Bachem (11), using repeated doses of over-all irradiation, found that "Many a radio-resistant tumor will disappear under divided dose therapy though it will not be affected by a massive dose of the same order of magnitude."

The significance of the time factor in repeated doses was emphasized by Jens Juul (8); and the importance of the time interval for a different phenomenon was shown by Kaplan and Brown (9), in reference to induction of lymphoid tumors in C57BL mice, where they demonstrate that "intervals of 4–8 days yielded a higher incidence, shorter latent period, and significantly deeper probit-regression line, indicating an optimal rhythm of exposure. Increasing the interval to 16 days resulted in a decreased response."

Our series of investigations, herewith reported, indicates that, under certain conditions of partial shielding, fractionated doses are more effective than acute doses.

In our experiments these conditions were obtained: (*a*) partial shielding of the animal and (*b*) fractionated radiation administered at 4-day intervals. Depopulation of the gonads of germinal cells and destruction of tumor cells were used as end-points for calculating quantitatively their radio-resistance.

The problem of induced radio-resistance presents itself in any study of radiation therapy by fractionated dosage and is especially pertinent to

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treatment of tumors. Thus, one of the specific aims of this project was to determine whether or not certain mouse tumors would acquire resistance to the injurious effects of radiation during a course of therapy.

At present, little is known about the fundamental nature of radio-resistance, either in normal or in tumor tissue. Windholz (13) gives a summary of widely held ideas as applied to tumors:

1. Each tumor consists of a conglomerate of resistant and sensitive cells (4, 12).

2. A biological mutation resulting in the formation of a new type of radio-resistant cell is induced by irradiation.

3. There is a change in the nature of connective tissue to the extent that it no longer hinders the relentless growth of tumor cells (10).

METHODS

Two strains of mice, maintained in the animal colony at Pondville Hospital, were used for this experimental work. Testes, ovaries, and the transplantable S 180 (10 days old) were taken from C57BL mice, and the spontaneous mammary adenocarcinoma (also 10 days old), from C3H mice. In total, 210 animals were used, 84 C57 males for the testes study, 66 C57 females for the ovary experiment, and 27 C57 mice with transplanted S 180 tumors, together with 33 C3H females bearing spontaneous mammary adenocarcinomas, for the tumor studies. In these experiments five doses of 294 r each were given at intervals of 4 days. The 4-day interval was chosen, since it had been demonstrated previously that following an acute dose of 300 r recovery was noted during this time. Factors for radiation were as follows: 100 kvP, 15 ma, 2.6 mm. Al HVL, 77 r/min at a TSD of 32 cm.

Each animal was restrained for irradiation by placing it in a rectangular-shaped celluloid box just large enough for the animal to enter. A small mesh wire cap was placed over one end to prevent suffocation. A plug of cotton in the open end, held in place by adhesive tape, prevented the animal from backing out of the box. Shielding was accomplished by placing lead plates over the mouse, leaving exposed only that anatomical region involving the organs in which we were interested—specifically, the testes or ovaries. When the tumors were irradiated, a similar procedure was followed, with only the tumor region left exposed while the rest of the animal was shielded.

Animals were killed at various time intervals. Those intervals which yield comparative data are indicated in Table 1.

TABLE 1

OUTCOME OF X-RADIATION ON SPERMATOGONIA OR SPERMATOCYTES IN MOUSE TESTES*

	Fractionated doses, 294 r each				Single dose, 300 r		Single dose, 1,440 r	
Doses	Days post-radiation	Days after first dose	Per cent tubules showing gonia	Per cent tubules showing cytes	Days post-radiation	Per cent tubules showing gonia	Days post-radiation	Per cent tubules showing gonia
1	4	4	36	79	4	40	4	24
2	2	6	9	82	6	11		
	4	8	0	74	8	16	8	6
							10	0
	8	12	6	37			12	1
		16	7	16				
3	2	10	0	68			10	0
	4	12	0	33			12	1
	8	16	0	14				
	10	18	1	0			18	2
	12	20	2	0	20	60		
4	2	14	0	0				
	8	20	0	0	20	60		
	21	33	8	0				
	42	54	14	6			54	26
5	2	18	0	0			18	2
	4	20	0	0	20	60		
	8	24	0	0				
	42	58	3	1			58	28

* Four animals killed at each time interval.

Tissues were fixed in Zenker's solution and sections stained in Harris' hematoxylin and eosin.

Five doses of 294 r[1] each, totaling 1,470 r, were used for comparison with the previously reported effects of acute doses of 300 and 1,440 r. In this work 294 and 300 r as well as 1,440 and 1,470 r are considered to be biologically equivalent. The quantity of radiation was measured with a condenser-type chamber, manufactured by the Victoreen Instrument Company. The percentage of inherent error is reported to be ±2 per cent.

OBSERVATIONS AND DISCUSSION

In the first series of experiments, quantitative data were obtained on the effects of fractionated doses (five doses, 294 r each, given at 4-day intervals and totaling 1,470 r) on the male germinal cells for comparison with previously reported data on animals killed at intervals after acute doses of

[1] Due to the physical conditions for irradiation, a dose of 294 r was used instead of 300 r as used in previous investigations.

300 or 1,440 r (5, 6). Four animals were killed at each of several intervals following each of the five doses. This sampling was considered adequate because of the consistent results achieved previously by our quantitative method (7).

A series of doses given at 4-day intervals did not change the pattern of response but produced a greater effect than an equivalent acute dose. This was reflected in a prolongation of the recovery period (Chart 1). Table 1 presents data on spermatogonia as well as corresponding data on spermatocytes for later discussion in connection with oocytes; these data on the spermatogonia do not include counts of the so-called prespermatogonia. It can be noted in Table 1 that a single dose does not bring about a complete temporary loss of spermatogonia. Any further irradiation does cause the depopulation, however. It is indicated that spermatogonia may reappear after any of the fractionated doses, but at successively later intervals.

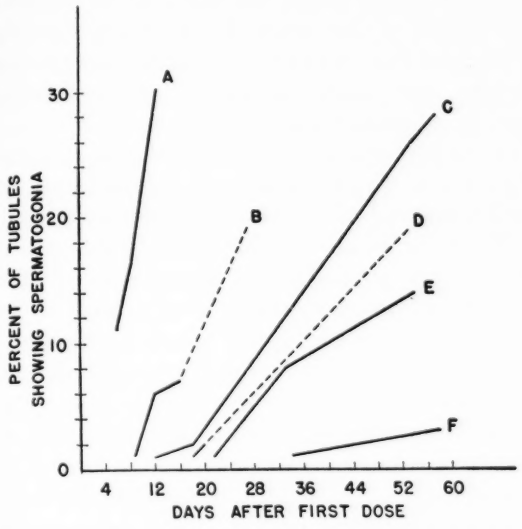

CHART 1.—Recovery of spermatogonia in mouse testes following various doses of x-radiation.

A.—300 r acute dose.
B.—588 r two doses.
C.—1,440 r acute dose.
D.—882 r three doses.
E.—1,176 r four doses.
F.—1,470 r five doses.

Dotted lines indicate recovery pattern.

Furthermore, in Table 1, by contrasting the effect of fractionated doses versus a single dose, at any given day postirradiation it can be seen that the fractionated doses are more effective. The above data further demonstrate that spermatogonia may reappear, although apparently complete depopulation has been accomplished, either by a fractionated or an adequate acute dose. It appears, therefore, that this recovery cannot be a case of multiplication of spermatogonia. The cytological studies confirm the observations of Barrow and Tullis (1) that repopulation comes from the capsular, elongate, resting cells, sometimes designated as prespermatogonia.

Apparently, the prespermatogonium is stimulated to multiply when there is an absence of spermatogonia. It is evident, also, that this function of the cell is not impaired by the amount of irradiation to which it has been subjected in these experiments. This observation lends further support to the suggestion of Tullis and Barrow (1) that prespermatogonia are relatively radio-resistant.

It should be possible, by our method of obtaining quantitative data, to express this radio-resistance as a numerical factor. It is indicated in the second column of Chart 2 that depopulation of the testes of spermatogonia may be accomplished at 4 days following the second dose (or 8 days after the first insult). It is indicated also, in Chart 2, column 3, that five doses do not eliminate the prespermatogonia. We do know, however, that prespermatogonia are destroyed by an acute dose of 5,050 r. These data are based on observations made 84 days after administering an acute dose (5). Observations, 180 days after administration of the dosage, still revealed no evidence of prespermatogonial activity, so that permanent inhibition of recovery was indicated.

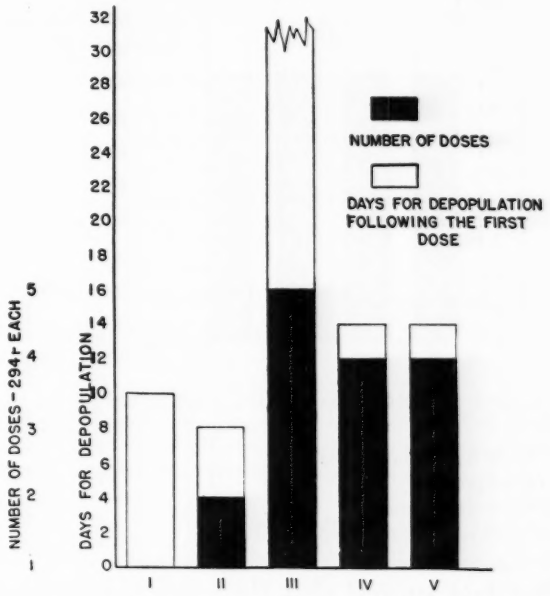

CHART 2.—Recovery of germinal cells following various doses of x-radiation.

I.—Spermatogonia, 1,440 r, acute dose.
II.—Spermatogonia, 588 r, two doses, 294 r each.
III.—Prespermatogonia, 1,470 r, five doses, 294 r each.
IV.—Spermatocytes, 1,176 r, four doses, 294 r each.
V.—Oocytes, 1,176 r, four doses, 294 r each.

Thus, it may be seen that prespermatogonia are more radio-resistant than sperm. Compare columns 1 and 2 in Chart 3. This difference in resistance to the effect of x-rays in prespermatogonia and sperm confirms observations of Barrow and Tullis and re-emphasizes their statement that "this presents an exception to the law of Bergonie and Tribondeau that primitive cells are more sensitive to ionizing radiation than specialized cells"(1).

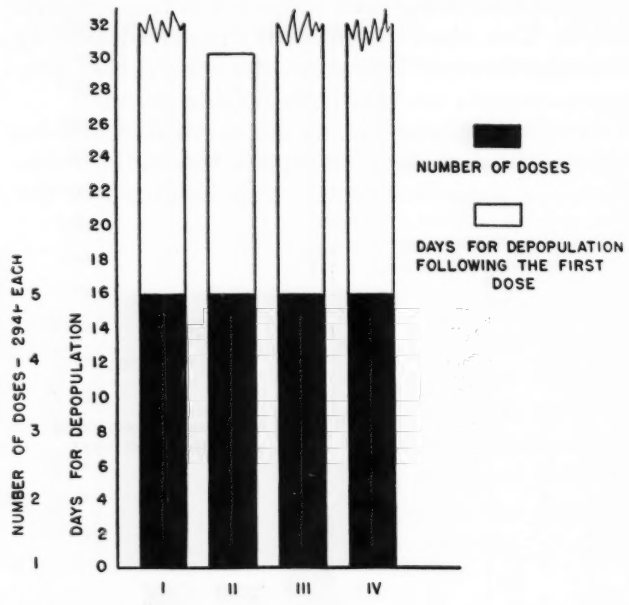

CHART 3.—Effect of fractionated doses on tumor and germ cells.

I.—Prespermatogonia.
II.—Sperm.
III.—S-180 tumor.
IV.—Spontaneous adenocarcinoma.

Our data suggest that there has been a basic, inherent, and irreversible change when the prespermatogonium becomes a spermatogonium. The nature of the change in the direction of increased radio-sensitivity from the prespermatogonial to the spermatogonial stage is not that which is commonly associated with morphological changes in a cell, such as progression to a prophase or active mitosis. This raises a question as to whether primitiveness, as defined by Bergonie and Tribondeau, and frequent mitotic activity can be considered the sole criteria for x-ray sensitivity either in normal or in neoplastic cells.

Two fractionated doses cause depopulation of spermatocytes of the testes (Table 1). These data are of interest, because they now make possible a direct comparison of the effect of x-radiation on analogous germinal cells in ovaries.

Female germinal cells.—The comparative sensitivity of germinal cells in the ovary and testis to x-radiation is summarized by Dunlap (2). In the testis the germinal epithelium is considered to be the most radio-sensitive element since the Sertoli cells, interstitial cells, and surrounding connective tissue survive doses of radiation that destroy germinal epithelium. In the ovary the ova and granulosa cells appear to be the most sensitive and the corpora lutea the most resistant. The stroma shows some progressive damage, as indicated by loss in weight of the ovary. Preliminary investigations suggest that oocytes and spermatocytes have relatively the same order of radio-resistance (Chart 2, columns 4 and 5).

The oocyte granulosa cells and the structural configuration which constitute a follicle are considered separately in evaluating effects of irradiation. The follicles are considered only as indicators for particular stages in the development of the oocyte. As is known, the early oocyte, as it appears in the primary follicle, is not much larger than its surrounding nurse cells. This is comparable to the early spermatocyte before growth has taken place. The oocyte rapidly increases in size, however, and by the time a medium-sized follicle is formed, the oocyte is as large as it will be prior to ovulation. The maximum-sized oocyte, as it appears in a medium-size follicle, seems to be the equivalent of the spermatocyte before meiotic changes occur. In this series of investigations, therefore, the medium-sized follicle was used for study. The criteria for determining effects of radiation were time and the number of doses (294 r each) necessary to cause break-down of the nucleus of the oocyte and change in configuration of the follicle. The term "depopulation" is used to describe the above situation as it was used previously to describe the loss of spermatocytes and spermatogonia.

A comparison between oocytes and spermatocytes is shown in Chart 2. Here it can be seen that by 2 days after the fourth dose of 294 r, medium-sized follicles are depopulated of oocytes, while the testis is devoid of spermatocytes. This would suggest that these germinal cells of ovaries have a factor of resistance which is not appreciably different from that of the corresponding cells of the testes. Further study is being made of this problem.

Evidence of a delay in recovery of the ovary following fractionation of dosage was observed in this study. The delay is much more extended for this organ than for the testis after identical doses. It may be recalled that a testis, depopulated of spermatocytes following a fractionated dose of 1,470 r, will begin to show the presence of spermatocytes at 42 days after the fifth dose (Table 1). The ovary, however, is devoid of oocytes for at least 3 months. The cause of this delayed recovery

in the ovary is unknown at the present stage of our studies.

Tumor.—The repeated dose procedure was applied to tumors according to the same methods and dosages as were used in the studies on male and female germinal cells. The tumors used were the transplantable S 180 and spontaneous mammary adenocarcinoma in C3H strain female mice.

It was noted that there were some cells in each of these tumors which equaled or exceeded in resistance the most resistant male germinal cells studied, the prespermatogonia (Chart 3). Microscopic observations suggest that these cells had been present in the tumors from the beginning, since, after each of the five doses, at any of the intervals studied, focal regions of typical tumor tissue could be found. In addition to the tumors for which results are illustrated, tumors of a few animals were subjected to ten and twelve doses (294 r each). These tumors, also, showed small focal regions of normal tumor tissue. Typical tissue was determined by the morphological appearance of the cells and the continued presence of cells in active mitosis with no evidence of any increase in abnormalities. Regions of normal tumor cells could be found at any interval following any of the doses, but, grossly, these regions became no larger or more numerous. This does not suggest induced resistance.

In brief, fractionated irradiation of tumors has shown that (*a*) there exist focal areas of typical tumor at all times and (*b*) these tumor cells are as resistant as the prespermatogonia. The interpretation of these results is not clear. It suggests that the opinion of Ferroux, Regaud, and Samssanow (4) that "each tumor consists of a conglomeration of resistant and sensitive cells" may have some factual basis.

If it is accepted that there is a varied population of cells in each of the tumors studied, in reference to radio-sensitivity, a comparison may be drawn between these tumors and the resistant male germinal cells as follows:

PRESPERMATOGONIA	TUMOR
Not visibly affected by the fractionated dose.	Some cells not visibly affected under the same conditions.
Differentiate into spermatogonia when occasion demands.	New tumor cells arise from the foci of resistant cells.
The spermatogonia are more radio-sensitive than the prespermatogonia.	These new cells seem to have the same sensitivity as did the original sensitive tumor cells.

It is known that the prespermatogonia can be destroyed if a sufficient dose is applied. It is assumed that the resistant tumor cells can also be destroyed, but it remains to be determined what dosage is required.

SUMMARY

Fractionated doses of 294 r produce a greater effect on spermatogonia and spermatocytes in partially shielded mice than does an equivalent acute dose.

After irradiation has caused depopulation of the tubules of spermatogonia, subsequent recovery is from capsular cells, called prespermatogonia.

The prespermatogonia are more resistant to radiation than are spermatogonia.

Oocytes and spermatocytes are relatively comparable in their response to doses of irradiation.

As compared to the testis, recovery following depopulation of germinal cells in the ovary is greatly delayed.

The fractionated dose required to depopulate the testis or ovary of germinal cells is not sufficient to cause destruction of all tumor cells in the tumors studied, namely, spontaneous mammary adenocarcinoma in C3H mice and the transplantable S 180 in the C57BL mice.

Apparently, induced resistance has not been achieved by repeated doses of irradiation under the conditions of our procedure.

ACKNOWLEDGMENTS

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Transient Regression of Murine Spontaneous Mammary Tumors with Analogs of Dimethyldiaminobenzene

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The rationale of the following work has been given in preceding papers (3, 4, 6). According to the views there proposed, it could be expected that antimetabolites of 1,2-dimethyl-4,5-diaminobenzene would be harmful to spontaneous mammary tumors of mice but not toxic to the animal as a whole. Provided that such an antimetabolite was not rapidly destroyed or excreted by the animal, the discovery of a selectively acting, tumor-destroying agent should be possible. The purpose of the present paper is to describe how some of the previously known antimetabolites of dimethyldiaminobenzene affect these spontaneous, malignant growths in mice.

The transposition of results found with bacteria to the problem posed in animals merits consideration. The known antimetabolites of dimethyldiaminobenzene have been observed to act on microorganisms (6). These antimetabolites have been separated from mere structural analogs of the metabolite (which are biologically inactive) on the basis of a test with *Staphylococcus aureus*. It is entirely possible that some which were inactive for this organism might be capable of functioning as antimetabolites in mammalian tissues. Similarly, animals might well be able to destroy or to excrete a potentially active compound, and thus to render it inoperative. Furthermore, the problem of getting an antimetabolite to the desired site of action in an animal and that of keeping it there are magnified in comparison to the related situation in microorganisms. Thus, one might anticipate that studies with bacteria would not lead without equivocation to the precise compound that would function as desired in animals. The best that could be expected is to be led to the general region of desirable effects.

METHODS

Animals.—Except where otherwise noted, the strain of Swiss mice maintained at this Institute was employed. Aging females occasionally develop relatively slowly growing mammary tumors which appear histologically as adenocarcinomas and which invariably lead to death (cf. 3). It has been previously noted that such growths appear to synthesize vitamin B_{12}, whereas normal animals apparently do not synthesize this vitamin. It was chiefly for this reason that this strain of mouse was used in the present work. The strain C mice bearing spontaneous mammary tumors that were studied came from the colony of Dr. P. Rous, through the kind co-operation of Dr. E. S. Rogers. Tumor-bearing C3H mice were purchased from the Jackson Memorial Laboratories.

Procedure for testing the ability of a substance to cause suppression of tumors.—Animals were selected when they exhibited palpable tumors. In a few instances the treatment was not begun until the neoplasms were large (e.g., 2.0–3.0 cm. long, 1.0–2.0 cm. wide, and 1.0–2.0 cm. high), but usually the growths were smaller. None was less than 0.5 cm. × 0.5 cm. × 0.3 cm. (Throughout this paper the last measurement refers to height.) The animals were caged individually and fed stock rations (bread, milk, and Purina Fox Chow). They were given daily (except Saturdays and Sundays) intraperitoneal injections of sterile neutral aqueous solutions or suspensions of the compound under study. The insoluble substances were prepared for injection by suspension of the powdered solid in physiological saline solution in a 50-cc. test tube containing four large glass beads. The tube was then rotated end-over-end, once per second, for 24 hours. The suspension was then heated to sterilize it. Control mice bearing similar tumors were given injections, in the same manner, of physiological saline solution.

The size of the tumors was measured at weekly intervals The condition of the animals and of their tumors was noted daily. Each animal was weighed once weekly.

In prior toxicity tests with normal mice the antimetabolites proved to be without detectable harmful effects or of relatively low toxicity. In fact, they had been designed with this end in mind (4–6). A dose of those compounds which did show toxicity was selected that would not cause loss of weight or outward signs of harm in the tumor-bearing animals.

RESULTS

Response of controls.—In the course of this study, 80 tumor-bearing mice were given injections of saline solution and were examined as described. The data in Table 1 reveal the distribution of the breeds, the average survival times, the range of survival times, and the number of transient regressions or transient obliterations of the tumors.

Transient regression and transient obliteration.—

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In this paper, the term "transient regression" of a tumor implies that for 1 or more weeks the dimensions of the tumor decreased. Following this interval, growth was resumed. "Transient obliteration" implies that the tumor shrank to the point where it could not be measured. At this stage it was not palpable, but usually some blemish of the skin remained so that one familiar with the animal in its cancerous state could just distinguish where the growth had been. Following this disappearance, the tumor began to grow once more, progressed to a large size, and caused the death of the animal. In no case was the obliteration permanent, although in a few of the treated animals it lasted for as long as 4 months.

Among the control animals, only two cases of transient obliteration were observed. In the more striking case a Swiss mouse was started on experiment when its tumor measured 2.0 × 1.0 × 1.0 cm. In 1 week it measured 1.0 × 0.5 × 0 cm., and at the end of the second week had shrunk to 0 × 0 × 0 cm. The tumor remained invisible for 3 weeks; then it began to grow again. One week later it measured 1.5 × 1.0 × 0.5 cm. Within 5 weeks it had attained a size of 4.0 × 4.0 × 3.0 cm., and the animal was discarded because of its moribund condition.

TABLE 1

PERFORMANCE OF MICE BEARING SPONTANEOUS MAMMARY TUMORS AND INJECTED DAILY WITH PHYSIOLOGICAL SALINE SOLUTION

Strain of mice	No. animals	Av. survival time (months)	Range of survival time (months)	Transient regressions	Transient obliterations
Swiss	36	2.4	0.3–7	1	1
C	29	2.1	1.5–3	0	1
C3H	15	1.6	0.8–3	2	0

Some judgment was required in assessment of a transient regression. If the decrease in size was found simultaneously with the ulceration of the tumor, it was considered insignificant. The opening of the skin and the formation of an ulcer occasionally resulted in a slight decrease in the height of the neoplasm and did not represent true regression. However, among the 80 controls, no diminution in height was observed to accompany the ulcerations. Such a change was observed only among treated animals.

Effect of 1,2-dichloro-4,5-diaminobenzene.—This substance was the first antimetabolite of dimethyldiaminobenzene to be recognized (4) and the first to be tried against spontaneous mammary tumors. It caused a slight response in that several of the tumors showed a transient regression. No transient obliterations were observed. Thus, eight C strain mice were given injections of 6 mg. of this compound per day, and, in addition, received it as 0.5 per cent of their food. Five similar mice served as controls. Three transient regressions occurred in the test group and none in the controls. Among seven C3H mice, similarly treated with the antimetabolite, there were three transient regressions, while in five controls there were two. The regressions were all of short duration, in that none exceeded 3 weeks. Although this effect was slight and its significance debatable, it encouraged trial with other compounds, especially when it was learned that mice readily destroyed this antimetabolite.

Destruction of 1,2-dichloro-4,5-diaminobenzene (DCDA) by mice.—A method for the quantitative estimation of 1,2-dichloro-4,5-diaminobenzene in the presence of mouse tissues was developed. It depended on the fact that alloxan reacts readily with this substance to give a fluorescent alloxazine. A similar reaction has been used as the basis of a method for the estimation of alloxan (1).

Mice injected with a saline suspension of 5 mg. of DCDA were each housed in a small beaker in order to collect any excreta. At the end of the desired interval of time, each animal was killed and blended in a Waring Blendor with 75.0 cc. of ethanol. The organic solvent probably stopped any enzymic destruction of the compound which might ensue, if it had not already done so, once the cells were broken. The suspension was filtered, and an aliquot of the clear filtrate, corresponding to one-quarter of the mouse, was concentrated under reduced pressure to a sirup, which was suspended in 20 cc. of 1 N HCl. The suspension was extracted 3 times with ether, and the extracts were discarded. The aqueous phase was then made alkaline with 10 cc. of 4 N NaOH and again extracted with ether. These extracts were concentrated under reduced pressure to a sirup which was dissolved in glacial acetic acid and made to 5.0 cc. Graded amounts of this solution, from 1.0 cc. to 0.1 cc. plus enough acetic acid to make the total volume 2.0 cc., were each treated with 1.0 cc. of a solution containing 50 mg. of alloxan, 50 mg. of boric acid, and 100 cc. of glacial acetic acid. Each mixture was heated in a boiling water bath for 3 hours, cooled, and examined for fluorescence under ultraviolet light. At the same time, a series of dilutions of DCDA ranging from 0.2 mg/tube to 0.02 mg/tube was similarly treated with the alloxan reagent. These were then used as standards. Interpolation on a standard curve gave values for the DCDA content of the unknowns. Extracts from mice injected with saline solution instead of with DCDA showed no detectable fluorescence under these conditions.

For recovery experiments normal mice were placed in a Waring Blendor with alcohol containing 5 mg. of DCDA, and, after blending, the operations were conducted as described. The extracts obtained contained approximately 5 mg. of DCDA. Thus, the compound could be recovered in satisfactory yield.

However, when the DCDA was injected into living mice it was rather rapidly destroyed. Thus, after 5 hours, less than 10 per cent of the 5 mg. introduced could be found by this procedure. This loss represented destruction, and not excretion, because the excreta were included in the sample. The alcohol used in the grinding of the sample was first used to wash out the beaker in which the mouse was kept after the injection.

Transient regression and obliteration of spontaneous tumors of mice treated with 1,2-dimethyl-4-amino-5-hydroxybenzene.—Experiments ranging from June, 1950, to July, 1952, consistently showed that the repeated injection of 1,2-dimethyl-4-amino-5-hydroxybenzene (dimethylaminophenol, DMAP) caused regressions and obliterations of tumors. This effect did not occur in all individuals treated, but either regression or complete obliteration was seen in 44 per cent. Furthermore, the mice were not cured of their neoplasm, because those which had disappeared eventually returned. In the case of strain C mice, two cases were seen in which the tumor was shed, leaving a massive crater. Loss of fluid from these wounds was large, infections were not controlled, and the animals died within a few days.

This antimetabolite of dimethyldiaminobenzene (6) was prepared according to the directions briefly suggested by Diepolder (2). The procedure employing dimethylnitrophenol was found impractical, but the one involving 1,2-dimethyl-4-phenylazo-5-hydroxybenzene gave excellent results.

The results with all mice tested are summarized in Table 2. It can be seen that 30 mice received the antimetabolite and that thirteen showed some suppression of their tumors. As mentioned in the table, two of these regressions were considered questionable.

Details of a few case histories illustrate more adequately the kinds of effects observed. The data of Table 3 show the fluctuations in the sizes of tumors of three mice which were affected and of two which were not. In these cases, and in every other instance in which regression was noted, the decrease began during the first few weeks of treatment. Usually, when the tumor began to decrease in size, the characteristic firm nature of the growth disappeared, and it became noticeably soft. The length of the period during which the tumor regressed was not the same in all animals but varied from 2 weeks in mouse 15 to 5 weeks in mouse 4. When the tumor began to grow again, it was characteristically firm.

Two of the C strain mice treated with DMAP shed their tumors, leaving massive craterous wounds. These cases were unprecedented. We have not observed this behavior in the controls of the C strain which we have had, and other investigators who have used this strain have informed us that they have never seen it. It was not found in any of our C3H or Swiss strain mice

TABLE 2

EFFECT OF REPEATED INTRAPERITONEAL INJECTIONS OF 1,2-DIMETHYL-4-AMINO-5-HYDROXYBENZENE (DMAP) ON SPONTANEOUS MAMMARY TUMORS OF MICE

Strain of mice	No. mice	Transient regressions	Transient obliterations	Daily dose (mg.)
Swiss	7	0	1	3
	13	4	1	5
	2	0	1	30 (oral)
C	5	2*	2†	5
	1	1‡	0	3
C3H	2	1	0	5

* One of these decreases in size occurred at the same time at which the tumor ulcerated. It was therefore of questionable significance. The other regression was bona fide.

† Tumors were shed, leaving large craters.

‡ This regression was questionable for the same reason given in *.

treated with DMAP, or with any other compound, or among untreated mice. Mouse 6 had a tumor 2.2 × 2.0 × 0.5 cm. when treatment was begun on December 21, 1951. One week later, the dimensions had increased to 2.6 × 2.3 × 0.8 cm. On January 4, 1952, the tumor had disappeared, leaving a large crater from which serum oozed. This wound began to granulate, but the mouse died on January 5. Mouse 36A had a tumor 2.0 × 2.5 × 1.0 cm. when treatment was started June 21, 1950. The crater was formed on July 5. Loss of fluid from this wound continued, and the mouse died on July 17.

In the untreated control animals of all strains the survival time varied markedly (cf. Table 1). It was therefore impossible to determine with certainty whether or not treatment with DMAP resulted in a lengthening of survival time. However, in so far as could be told, there was no significant increase. Once the tumors had resumed growth they frequently progressed rapidly.

Destruction of 1,2-dimethyl-4-amino-5-hydroxybenzene (DMAP) in mice.—In order to determine whether or not the failure of all mice to respond was associated with individual differences in abil-

ity to destroy DMAP, a quantitative method was developed for the estimation of this compound in mouse tissues. This method was based on the fact that *o*-aminophenols give a yellow color when treated in ethanolic solution with salicylaldehyde. A fluorescence also developed, but the color proved to be a better quantitative guide.

Individual mice were each injected intraperitoneally with an aqueous suspension of 5 mg. of DMAP. Excreta were collected as described in the procedure for the determination of DCDA. After various time intervals, the animals were killed and extracted. The procedure was the same as that described above for DCDA, except that 10 cc. of 1 N HCl was used to suspend the aliquot of mouse extract prior to the ether extraction. Following this extraction the aqueous phase was concentrated under reduced pressure to a sirup and diluted to 5.0 cc. with ethanol. The DMAP could not be extracted quantitatively into ether from either alkaline or neutral solutions, and it was therefore measured in the aqueous phase. Graded amounts of the final alcoholic sample, plus enough ethanol to make a volume of 8.0 cc., were placed in Evelyn colorimeter tubes and treated with 2.0 cc. of a solution prepared from 1.0 cc. of salicylaldehyde, 95.0 cc. of ethanol, and 5.0 cc. of glacial acetic acid. At the same time a series of standards was set up with amounts of DMAP ranging from 0.1 mg. to 0.01 mg/tube. The colors were then measured photoelectrically at a wave length of 420 mμ. From the calibration curve constructed with the aid of the standards, the values for the unknowns were calculated. A typical standard curve is shown in Chart 1.

Recovery experiments done, as with DCDA, by addition of DMAP to the alcohol in which a normal mouse was subsequently ground, showed that, when 5 mg. of DMAP was added, 4.8–5.4 mg. were recovered. Blanks done on extracts of mice not injected with DMAP showed less than 0.5 mg. of this substance, or interfering chromogen calculated as it, per mouse. However, because when DMAP was stored in solution it rapidly turned brown, all operations were conducted with utmost speed and the analysis was completed in 1 day.

Analyses of several adult mice, each of which had received 5 mg. of DMAP, showed that all the compound could be recovered if the animals were killed and extracted immediately after injection. When 5 hours elapsed between injection and analyses, 12–30 per cent could still be found.

Differences among individuals of a single strain of mice in extent of destruction of dimethylaminophenol (DMAP).—Analyses performed in the way just described on eight individual normal Swiss strain mice injected intraperitoneally with 5 mg. of dimethylaminophenol showed that, although these mice were all from the same strain, they were not identical with respect to their ability to destroy this substance (Table 4). More than half of the animals were found to destroy 80–88 per cent (i.e., only 10–20 per cent, or 0.5–1.0 mg. was recovered), while others were able to dispose of only 70 per cent in the same period of time.

Does the variation in capacity to destroy dimethylaminophenol explain why only some tumors respond to treatment with this substance?—The hypothesis might be formed that some individuals, because of increased capacity to destroy DMAP, did not retain sufficient amounts of it to result in suppression of the tumor. This hypothesis might be strengthened by the data of Table 4. More than half of the animals destroyed 80–88 per cent. It will be recalled that among tumor-bearing Swiss mice approximately the same proportion were not benefited by DMAP. However, to the present

TABLE 3

VARIATIONS IN SIZES OF SPONTANEOUS MAMMARY TUMORS OF SWISS MICE DURING TREATMENT WITH 1,2-DIMETHYL-4-AMINO-5-HYDROXYBENZENE (DMAP)

Time interval (weeks)	Mouse 20 March, 1951 (cm.)	Mouse 12 January, 1952 (cm.)	Mouse 4 Starting date December, 1951 Tumor measurements (cm.)	Mouse 29 February, 1952 (cm.)	Mouse 23 February, 1952 (cm.)
0	1.5×1.0×0.5	2.0×1.3×0.3	1.6×1.5×1.8	1.0×2.0×0.3	1.6×1.4×0.4
1	1.0×1.0×0.5	2.0×1.5×0.3	2.5×2.1×2.0	1.2×2.9×0.4	2.0×1.9×0.5
2	1.0×1.0×0.5	2.0×1.3×0.3	2.5×2.3×1.2	1.4×3.0×0.9	2.0×1.9×0.5
3	0×0×0	1.9×1.3×0.3	2.2×2.2×0.5	1.5×3.0×1.0	2.0×2.0×1.0
4	0×0×0	1.9×1.3×0.1	2.0×1.8×0.2	2.0×3.2×1.0	2.0×2.5×1.0
5	0×0×0	1.9×1.1×0.1	2.0×1.5×0.1	2.5×4.0×1.7	2.3×3.0×1.0
6	0×0×0	1.5×1.5×0.1	1.0×1.0×0.2	3.0×4.0×1.7	3.0×3.2×1.3
7	0.5×0.5×0.5	2.0×1.8×0.1	1.3×1.4×0.9	dead	3.3×3.8×1.9
8	0.5×0.5×0.5	2.0×1.9×0.3	1.8×1.5×1.0		
9	0.5×0.5×0.5	2.5×1.9×0.3	2.0×2.0×1.0		
10	0.5×0.5×0.5				

author, it seemed that the difference in capacity to destroy DMAP was not great enough to account for the failure to affect all the tumors. This conclusion was supported by the following experiment: Four Swiss mice bearing spontaneous mammary tumors were fed a diet of powdered Fox chow plus 1 per cent DMAP. Food intake was measured, and it was found that each mouse ingested about 30 mg. of the antimetabolite per day. Nevertheless, these animals, which were receiving 6 times as much as those previously described,

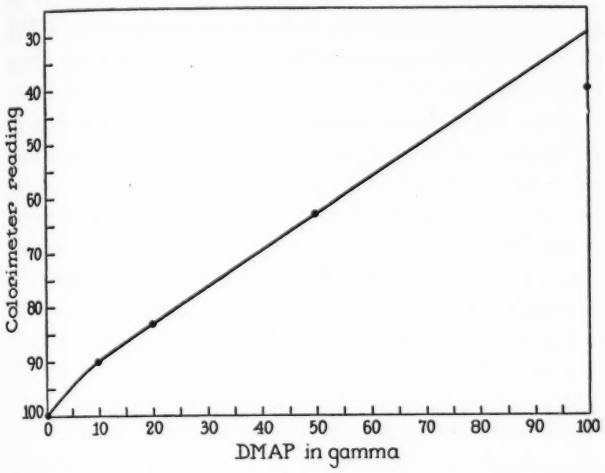

CHART 1.—Typical standard curve for the colorimetric estimation of DMAP.

showed no greater incidence of favorable effect on the suppression of their tumors. Only one was decreased in size. It was not known, however, whether the oral route was equal to the intraperitoneal when the same dose was administered.

These considerations do, nevertheless, show that recognizable quantitative differences do exist among individuals of a single strain. The failure of all members of this strain to be benefited by DMAP might then be understandable—if not in terms of this specific function, perhaps in terms of some other.

Does the transient character of the tumor regression caused by DMAP result from an increasing capacity of the mouse to destroy the beneficial substance?—The following experiments showed that the return of the tumors was probably not due to the acquisition by the mice of greater capacity to destroy DMAP. Each of four mice which had exhibited regression of their tumors under the influence of DMAP, but which had subsequently exhibited regrowth at the old sites, was tested in the manner just described for its ability to destroy a 5-mg. dose of DMAP in 5 hours. Because these mice had been treated constantly with the antimetabolite, this treatment was discontinued for 4 days prior to the test dose. This was done to allow the mouse to dispose of DMAP before being challenged again. Results of this study are shown in Table 5. These animals had no abnormally large capacity to rid themselves of the antimetabolite. In fact, they were all below the average of the eight mice of Table 4. This last point might be used in support of the idea that only those mice which destroyed DMAP poorly were benefited. It will be remembered that these mice of Table 5 had each undergone a regression of a tumor under the influence of DMAP.

TABLE 4

AMOUNTS OF DMAP RECOVERED FROM VARIOUS INDIVIDUAL MICE OF THE SWISS STRAIN (PLUS EXCRETA) INJECTED WITH 5 MG. OF DMAP

Mouse no.	DMAP recovered (mg.)	Per cent destruction
E	1.5	70
G	0.6	88
H	0.6	88
I	1.0	80
J	1.5	70
K	1.2	76
L	0.8	84
14A	0.7	86

TABLE 5

DMAP-DESTROYING ABILITY OF TUMOROUS SWISS MICE, THE TUMORS OF WHICH HAD RELAPSED FOLLOWING A REGRESSION CAUSED BY DMAP

Mouse no.	Type of regression	Time from relapse* to analysis (weeks)	DMAP recovered (mg.)	Per cent destruction
4	Transient regression	4	1.1	78
14	Transient regression	4	1.8	64
28	Transient obliteration	3	2.0	60
30	Transient obliteration	2.5	1.2	76

* This interval was taken from the time at which the regressed tumor began to grow again until the mouse was used for the experiment.

Unsuccessful attempts to obtain permanent obliteration of tumors with DMAP plus a second compound.—Six Swiss mice bearing spontaneous mammary tumors were used in these trials. Four were given daily injections (except Saturdays and Sundays) of 5 mg. of DMAP and 1.5 μg of aminopterin. The other two received DMAP plus 1 mg. of 2,6-diaminopurine per day. There were one obliteration and one regression of a tumor in the first group which followed the typical course outlined for animals getting only DMAP. The tumor reappeared and grew rather rapidly. The mouse

which had shown the regression died. Both mice which received DMAP plus 2,6-diaminopurine failed to show regressions. Clearly then, these additional treatments did not prevent relapses. Neither did they make all the tumors respond.

Transient obliteration of spontaneous mammary tumors caused by 1,2-dimethyl-4-(p-carboxyphenylazo)-5-hydroxybenzene plus 1,2-dichloro-4-(p-nitrobenzenesulfonylamido)-5-nitrobenzene. — Because the relapses on DMAP, as well as the failure of all tumors to respond, might have been due to increased production in some mice of the metabolite dimethyldiaminobenzene, which nullifies the action of the antimetabolite (6), the aggregate analogs of dimethyldiaminobenzene and of *p*-aminobenzoic acid were investigated. These aggregate analogs were developed for this reason, and their actions on bacteria have been described recently (5). In microorganisms the harmful effect of these aggregates was not antagonized by the related metabolites, and the same might be true for tumors.

Swiss mice bearing spontaneous mammary tumors were handled as described in the experiments with DMAP and were injected daily with a sterile, neutral aqueous solution containing 2 mg. of 1,2-dimethyl-4-(*p*-carboxyphenylazo)-5-hydroxybenzene and 0.2 mg. of 1,2-dichloro-4-(*p*-nitrobenzenesulfonylamido)-5-nitrobenzene. These substances were called carboxyphenylazo (CPA) and dichlorodinitrosulfa (DCDNS) for the sake of brevity. They were prepared as previously described (5).

The course of events was similar to that described for mice treated with DMAP. During the 2d or 3d week of treatment several of the tumors became markedly soft in texture and began to decrease in size. Within 2 or 3 weeks complete obliteration of some was seen. With others, the tumors regressed to a rather small size, but remained measurable, being about 0.2–0.3 cm. high. These then remained quiescent for from 1–4 months. Both the obliterations and the regressions were of distinctly longer duration than was the case with DMAP. All tumors, whether they had exhibited obliteration or regression, eventually began to grow again.

Of the fourteen animals treated with this mixture of aggregate analogs, two showed obliterations and five had regressions. Detailed records of four of these mice are shown in Table 6, which illustrates the various types of response.

Effect of 1,2-dimethyl-4-(p-carboxyphenylazo)-5-hydroxybenzene (CPA) alone.—In order to determine whether one of the aggregate analogs alone would be effective, four tumor-bearing Swiss mice were given injections daily (except Saturdays and Sundays) of 2 mg. of CPA. None showed any regression in the size of the tumors.

Four similar mice were fed a ration composed of fox chow containing 1 per cent CPA plus 0.4 per cent of $NaHCO_3$. One of these exhibited obliteration of the tumor. The regression began 3 weeks after the start of the experiment, and the tumor again became measurable in the 7th week of the test. Among five similar mice fed only fox chow, no regressions were observed.

Although the number of animals involved in these experiments was small, the mixture of CPA plus DCDNS appeared to be more effective than CPA alone.

Was the failure of all tumors to respond due to differences in type of neoplasm?—Biopsy specimens were taken from the four mice which were to be fed the CPA as described in the preceding experiment. These specimens were taken at the beginning of the trial and were examined histologically through the courtesy of Dr. H. B. Andervont who reported that each was a mammary adenocarcinoma. Because one tumor responded to treatment with CPA and the other three did not, it was plain that all mammary adenocarcinomas of Swiss mice would not respond. Prior to this trial it might have been argued that, although each mouse carried a tumor, it was not necessarily of the mammary adenocarcinoma class.

Attempts have been made to correlate the position of the tumor on the body of the mouse with the response or lack of response to the compounds studied in this work. These attempts have not yet proved successful. In general, it has appeared that tumors which were small (e.g., $1.2 \times 1.0 \times 0.4$ cm., mouse 66) at the start of treatment were more likely to regress than were very large structures. However, by no means did all small tumors respond.

Toxicity of the antimetabolites.—As described previously (5), DCDNS was toxic to normal mice when large doses were injected. Therefore, in the present investigation, the daily dose was low enough to avoid readily discernible harmful effects. CPA was well tolerated by normal mice (5). The mixture of CPA plus DCDNS used in the present study did not cause loss of weight. It did seem to cause some sensation immediately after injection, because the mice would scratch the site of injection for a short time after the needle was withdrawn. A few animals developed a patchy erythema of the skin of the face after they had been receiving this mixture for many weeks. Weanling mice which were injected daily with 1 mg. CPA plus 0.1 mg. of DCDNS grew at the same

rate as those similarly injected with physiological saline solution (36 mice in each group).

No toxic manifestations were noted in normal adult mice injected daily with 6 mg. of DMAP. Likewise, the addition of 1 per cent of this compound to a fox chow diet caused no detectable harmful effects in adult mice which consumed it. Weanling mice injected with 1 mg/day grew at a rate approximately equal to that of similar animals injected with saline solution (nineteen mice in each group; period of observation, 2 months). Weanling mice which received daily injections of 5 mg. of DMAP grew at subnormal rates. After several weeks, these animals showed other signs of damage, the most noticeable of which was bleeding around the eyes.

Observations were limited to these gross findings. No histological examinations were made. However, normal litters were repeatedly obtained from mice during a course of injections of either DMAP or CPA plus DCDNS.

TABLE 6

VARIATIONS IN SIZES OF SPONTANEOUS TUMORS IN SWISS MICE INJECTED DAILY WITH 1,2-DIMETHYL-4-(*p*-CARBOXYPHENYLAZO)-5-HYDROXYBENZENE (CPA) PLUS 1,2-DICHLORO-4-(*p*-NITROBENZENESULFONYLAMIDO)-5-NITROBENZENE (DCDNS)

	Mouse 65	Mouse 66	Mouse 81	Mouse 67
	Starting date			
Time interval (weeks)	May, 1952	May, 1952	June, 1952	May, 1952
	Tumor measurements			
	(cm.)	(cm.)	(cm.)	(cm.)
0	2.4×2.0×1.0	1.2×1.0×0.4	1.0×1.5×0.5	2.0×1.0×0.3
1	2.4×2.1×0.3	1.5×1.5×0.8	1.0×1.5×0.5	2.3×1.3×0.3
2	1.5×1.2×0.2	1.1×1.4×0.6	1.0×1.0×0.4	2.3×1.6×0.3
3	0×0×0	1.0×1.0×0.3	0.5×0.5×0.3	2.3×1.9×0.5
4	0×0×0	0×0×0	0.5×0.5×0.3	2.4×2.0×0.6
5	0×0×0	0×0×0	0.5×0.5×0.3	2.5×2.0×0.8
6	0×0×0	0×0×0	0.5×0.5×0.3	2.9×2.2×1.0
7	0×0×0	0×0×0	0.5×0.5×0.3	3.0×2.2×1.0
8	0×0×0	0×0×0	0.5×0.5×0.3	3.0×2.5×1.0
9	0×0×0	1.3×1.0×0.3	0.5×0.5×0.3	3.3×3.1×1.0
10	0×0×0	2.0×1.2×0.3	0.5×0.5×0.3	3.5×3.3×1.2
11	0×0×0	2.1×1.4×0.3	0.5×0.5×0.3	3.5×3.3×1.2
12	0×0×0	2.2×1.4×0.3	0.5×0.5×0.3	3.5×3.3×1.2
13	0×0×0	2.3×1.5×0.3	0.5×0.5×0.3	3.5×3.3×1.3
14	1.5×1.5×0.4*	2.8×2.8×0.9	0.5×0.5×0.3	3.5×3.3×1.3
15	1.5×1.5×0.4	3.0×3.5×1.3	0.5×0.5×0.3	3.8×3.5×1.5
16	1.5×1.5×0.4	3.5×4.0×1.5	0.5×0.5×0.3	4.5×4.0×1.5
17	1.5×1.6×0.4	5.0×4.5×2.0		5.0×4.0×2.0
18	1.5×1.6×0.8			5.0×4.5×2.0
19	2.0×1.8×0.8			
20	2.0×1.8×0.8			

* A second tumor appeared which grew relatively rapidly. Dimensions of this are not given.

DISCUSSION

Although DMAP and CPA plus DCDNS have given more favorable results with *spontaneous* mammary tumors than have other substances previously known, the degree of success achieved was unsatisfactory. In the first place, all individuals did not respond favorably. No more than half showed either regression or obliteration. In the second place, the regressions and obliterations were invariably of a temporary character.

The apparent acquisition by the tumor of resistance to the drugs would seem to be a major unsolved problem besetting the present approach to the chemotherapy of cancer. One may view the transient nature of the suppressions as being attributable to the persistence of viable malignant cells. From this remnant population, possibly by mutation, one could picture a variant as being slowly selected, which then grew vigorously in the presence of the compound which once caused the downfall of most neoplastic neighbors. On the other hand, the resistance may arise from acquisition by the host animal of an ability to inactivate the antimetabolites. The data are insufficient to substantiate this mechanism, but, by some, might be construed in support of it.

Because all individuals were not affected by DMAP or by CPA plus DCDNS, and especially because a few of the controls exhibited regression, we may well inquire whether the compounds actually did exert an effect. A calculation by an experienced statistician showed that the probability of the result on all mice treated with DMAP being due to chance was less than 1 in 1,000. A similar calculation for the effect of CPA plus DCDNS gave P approximately 0.001.

The compounds used in this study were considered to be antimetabolites of 1,2-dimethyl-4,5-

diaminobenzene. Evidence that they are has been obtained previously with *Staphylococcus aureus* (5). No data are available to show whether or not the effects in animals are of the same character. Because DCDA was so readily destroyed by mice, it was considered probable that dimethyldiaminobenzene would also be metabolized rapidly and for the same reason. Therefore, attempts to reverse the effects of DMAP in tumorous animals did not seem promising, especially since mice with *spontaneous* tumors were rare and needed for other purposes.

It is important to emphasize that these observations have been made on spontaneous tumors. Transplants have not been used. In view of findings previously reported (3), the failure to duplicate these observations in transplanted tumors would not be too surprising.

SUMMARY

1,2-Dimethyl-4-amino-5-hydroxybenzene (DMAP), an antimetabolite of 1,2-dimethyl-4,5-diaminobenzene, caused some spontaneous mammary tumors of Swiss or of C strain mice to decrease in size or to disappear. More than half of the treated animals were not thus affected by this substance. In most of the affected animals the tumor decreased in size but did not disappear. In every instance of regression, the effect was transient, and eventually the tumor grew once more and killed the host. The DMAP was not detectably toxic to adult mice. A satisfactory quantitative method for the estimation of DMAP in mouse tissues was described. Mice were found to destroy or to excrete DMAP injected into them, but 12–40 per cent of a single dose could be recovered from a mouse 5 hours after administration. Individual mice of the single Swiss strain were observed to differ with respect to the percentage of an injected dose which they destroyed. Although the proportion of mice actively destroying DMAP corresponded roughly to the proportion of tumorous mice which did not respond to this compound, the magnitude of the difference in capacity to destroy DMAP was not considered great enough to explain completely why some tumors were caused to regress and others were not. The reappearance of obliterated tumors was not associated with the acquisition of great ability to destroy DMAP. However, all mice in which tumors had been caused to regress by injection of DMAP showed less than average capacity to destroy the drug.

A barely perceptible effect in causing regression of spontaneous mammary tumors in C and in C3H mice was observed with another antimetabolite of dimethyldiaminobenzene, viz., 1,2-dichloro-4,5-diaminobenzene. A quantitative method for the estimation of this compound in mouse tissues was developed. The use of this method revealed that mice readily destroyed this substance.

One-half of a group of Swiss mice bearing spontaneous mammary tumors exhibited either a decrease in size or a complete obliteration of the tumors when the animals were injected daily with a mixture of 1,2-dimethyl-4-(*p*-carboxyphenylazo)-5-hydroxybenzene and 1,2-dichloro-4-(*p*-nitrobenzenesulfonylamido)-5-nitrobenzene. These aggregate analogs of dimethyldiaminobenzene and *p*-aminobenzoic acid were not able to cause permanent destruction of the tumors, because those which had been obliterated reappeared after several months.

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Immunological Properties of Carcinogen-Protein Conjugates Containing 4-Dimethylaminostilbene and Its 2′-Methyl Analog*

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This paper presents data on the immunizing capacity and prosthetic group activity of conjugates prepared by the interaction of serum albumins and the 4′-isocyanates of 4-dimethylaminostilbene and its 2′-methyl analog. Such information was required for studies of the possibility of immunizing animals against systemic carcinogenesis. The detailed objectives, background information, preparative and analytical procedures, as well as some of our serological results, have been reviewed recently (1).

EXPERIMENTAL

The conjugates were prepared, purified, and analyzed spectrophotometrically as described by Creech and Peck (3), using an aqueous dioxane medium for the combination of the proteins with the stilbenyl isocyanates (8, 9). The designations for the conjugates show the type and number of prosthetic groups attached to horse serum albumin (HSA) and bovine serum albumin (BovA). The symbol *S* represents the 4-dimethylaminostilbenyl-4′-carbamido group, $(CH_3)_2NC_6H_4CH$:-$CHC_6H_4NHCONH$-, and 2′-MeS represents the 2′-methyl-4-dimethylaminostilbenyl-4′-carbamido group, $(CH_3)_2NC_6H_4CH:CHC_6H_3(CH_3)NH$-$CONH$-. The number of groups is given in the final part of the symbol; thus, S-HSA-15 stands for a horse serum albumin conjugate containing fifteen 4-dimethylaminostilbenyl-4′-carbamido groups per molecule of protein.

The antigens employed in the study were HSA, S-HSA-4, S-HSA-15, S-HSA-26, S-HSA-34, 2′-MeS-HSA-14, 2′-MeS-HSA-55, BovA, S-BovA-20, S-BovA-31, 2′-MeS-BovA-13, 2′-MeS-BovA-37, and 2′-MeS-BovA-62. The use of carefully prepared conjugates containing widely different numbers of prosthetic groups permits critical evaluation of the influence of foreign components on the immunological properties of proteins.

Antisera were obtained from 5- to 7-month-old albino rabbits which had been injected intraperitoneally 3 times a week with a total of 216 mg. of antigen as follows: 8 mg. in 1 ml. of physiologic saline per injection in the 1st week, 16 mg. per injection in the 2d week, and 24 mg. in the 3d and 4th weeks. Groups of two to five rabbits were injected with antigen after the serum of each rabbit had been tested to exclude any reaction due to naturally occurring antibodies. The rabbits were test-bled 1 day after the last injection. If the antibody level was low, an additional 3-week course of injections with a total of 192 mg. of antigen was given. The rabbits remained healthy during the period of immunization. They were exsanguinated by heart puncture 5 days after the final injection. The antisera were stored without a preservative in a deep freezer at −10° F., pooled appropriately, and used immediately after thawing.

The antigen-antibody reactions were studied by micro-Kjeldahl analyses of the washed precipitates, according to the quantitative precipitin technic of Heidelberger and Kendall (4). Depending on the results of trial runs, 0.5–2.0 ml. of an antiserum was allowed to react with amounts of antigen ranging from 0.4 to 3.0 mg/ml of serum. Each determination was run in duplicate, and controls containing serum and saline, as well as antigen and saline, were tested. Twenty-four runs were usually made at one time. The region of maximum precipitation was generally located between the equivalence zone (determined by adding antigen and antibody to aliquots of the supernatant fluid from an antigen-antibody reaction) and the region of antigen excess.

Preliminary trials were conducted to obtain information on the probable optimum time for exsanguination of the immunized rabbits. Heidelberger, Kendall, and Soo Hoo (5) recommend bleeding animals on the 5th day, whereas Marrack (7) believes that bleeding should not be done before the 10th–12th day, because of persistence of antigen in the blood stream. In earlier work with hydrocarbon-protein conjugates, Creech, Oginsky, and Cheever (2) exsanguinated the rabbits either after 10–13 days or after 17–20 days, depending on the procedure of immunization. Several rabbits were immunized with 2′-MeS-HSA-55 or with 2′-MeS-BovA-62, and 50 ml. of blood was obtained by sterile heart puncture 5, 10, 16, and 27 days after the final injection. The amount of antigen-antibody precipitate, produced at the equivalence zone by the addition of 0.8 mg. of homologous antigen to 1 ml. of antiserum, showed an over-all decrease of 74 per cent from day 5 (2.1 mg. of precipitate) to day 27 (0.54 mg. of precipitate) in the case of the antiserum toward 2′-MeS-HSA-55. A decrease of 32 per cent in the same period was noted with the antiserum

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toward 2′-MeS-BovA-62. Although not conclusive because of the heavy test bleeding, this indicated the desirability of bleeding the rabbits on the 5th day after the final injection. Satisfactory antibody levels were generally noted 1 day after the final injection.

Because of the absorption maximum displayed by the stilbenyl group of the conjugates (3), it was anticipated that spectrophotometric analysis of an aliqout of the washed antigen-antibody precipitate in alkaline aqueous dioxane solution, together with a nitrogen analysis on the remainder of the precipitate, would readily permit determination of the ratio of antibody to antigen. It was noted, however, that exposure to light of the antigen-antibody complexes in alkaline solution had a pronounced effect in decreasing the intensity of absorption of the stilbene molecule (cf. 3). The molecular ratios of antibody to antigen in the homologous protein series usually varied from 3:1 near the equivalence zone to 1:1 at antigen excess; lower values were noted with test conjugates containing heterologous protein. The true ratios must be lower, however, because the complex contained more antigen than was indicated spectrophotometrically. Another attempt will be made to obtain more precise values.

TABLE 1

CHANGES OCCURRING WITH TIME IN SOLUTIONS OF THE CONJUGATES

Conjugate	Per cent decrease in nitrogen value	Per cent decrease in intensity of absorption (based on nitrogen value of the supernatant fluid)	Shift in absorption maximum
HSA	5		
S-HSA-4	3	47	355–330 mμ
S-HSA-15	0	15	None
S-HSA-26	1	5	None
S-HSA-34	14	31	None
2′-MeS-HSA-14	3	57	355–315 mμ
2′-MeS-HSA-55	18	10	None
BovA	3		
S-BovA-20	21	17	None
S-BovA-31	0	10	None
2′-MeS-BovA-13	13	39	355–350 mμ
2′-MeS-BovA-37	4	10	None
2′-MeS-BovA-62	36	34	None

Since this discovery was made after about one-half of the antigen-antibody determinations had been performed, all the several-month-old stock solutions of test antigens were reexamined to ascertain if any *trans-cis* shift (3) in the stilbene configuration had accompanied the drop in intensity. An appreciable amount of such a transformation would be expected to have an influence on the amount of antibody-antigen precipitate, because geometric isomers such as maleic and fumeric acids can be differentiated by the use of appropriate antisera, as found by Landsteiner and van der Scheer (6).

The observations made on 2- to 7-month-old solutions originally containing 4 mg of conjugate/ml of isotonic saline are presented in Table 1. The solutions were centrifuged at 5,000 r.p.m. prior to analysis of the supernatant fluid. It will be seen from the nitrogen values that the most unstable solution was that of the high carcinogen-containing antigen 2′-MeS-BovA-62 which often became cloudy in a few weeks. This conjugate had to be prepared frequently by dialysis of stock preparations stored under 2 M ammonium sulfate; solutions employed in the serological studies were generally less than 2 months old. The conjugates S-HSA-34, 2′-MeS-HSA-55, S-BovA-20, and 2′-MeS-BovA-13 tended to become opalescent on long standing and occasionally required centrifugation before use. In some instances, then, these five test antigens had been added to the antisera in amounts which may have been 10–15 per cent less than indicated in the abscissae of the graphs. Aqueous solutions of the other test antigens remained optically clear for several months.

The third column of the Table shows the occurrence of appreciable decreases in the intensity of absorption of the stilbene component of the conjugates in the supernatants of S-HSA-4, S-HSA-34, 2′-MeS-HSA-14, 2′-MeS-BovA-13, and 2′-MeS-BovA-62. Measurements on S-HSA-4 are not particularly accurate because of the low content of stilbene. Although S-HSA-34, 2′-MeS-HSA-14, and 2′-MeS-BovA-13 were prepared in low actinic glassware (3), there is more involved than a delayed action of light, because 2′-MeS-BovA-62 was prepared in standard glassware. Moreover, since the latter more unstable conjugate was also found to be electrophoretically homogeneous (9), the drop in intensity of absorption of the portion of conjugate in the supernatant fluid did not result from preferential denaturation and precipitation of material with a higher than average content of stilbene component.

The last column of Table 1 shows that there was a shift in the absorption maximum (indicating appreciable *trans-cis* change) in only three of the preparations—namely, S-HSA-4, 2′-MeS-HSA-14, and 2′-MeS-BovA-13, all of which exhibited low prosthetic group activity. Decreases in intensity also occurred in the stock preparations of three of the conjugates during storage under 2 M ammonium sulfate for a period of approximately 1 year. Spectrophotometric analyses of fresh solutions of these conjugates obtained by dialysis in the absence of light showed decreases in the intensity of absorption (without any shift in the wave length of the absorption maximum, however) as follows: 2′-MeS-HSA-14, 9 per cent; S-HSA-34, 9 per cent; 2′-MeS-BovA-62, 11 per cent.

To determine any influence of the drop in intensity of absorption of the conjugates on their activity as test antigens, comparisons were made between solutions which had been kept in the dark and those which had been deliberately exposed to light for 20 hours. Some of the results are presented in Table 2. The letter *D* attached to the symbol for the test antigen indicates the preparation kept in the dark, and *L* indicates the antigen

exposed to light. In each of the latter cases, there was a decrease in intensity of absorption of about 30 per cent. Surprisingly, the amount of antigen-antibody precipitate produced by addition of the light-exposed conjugates to the antisera toward homologous carcinogen-heterologous proteins used in this study was about 15 per cent greater than that produced by the conjugates which had been kept in the dark. It is evident, however, that the changes which occurred in the stilbenyl prosthetic group of the conjugates upon exposure to light did not appreciably influence its serological activity.

The results of the micro-Kjeldahl analyses of the washed antigen-antibody precipitates are presented in graphical form for greater ease of comparison. The curves are plotted on the basis of the number of milligrams of antigen-antibody precipitate obtained by the addition of three to six different levels of antigen per milliliter of antiserum. Each point on the graph represents the average value of two to six determinations made on pooled antisera. In about 80 per cent of the cases, we were successful in locating either the zone of maximum antigen-antibody formation or the region where the curve was rising only slightly at antigen excess. The test antigens containing the highest number of prosthetic groups, 2′-MeS-HSA-55 and 2′-MeS-BovA-62, accounted for the majority of the exceptional cases. Shortages of antisera prevented completion of these curves.

TABLE 2

EFFECT OF LIGHT ON THE ACTIVITY OF TEST ANTIGENS

Mg of antigen-antibody precipitate/ml of antiserum

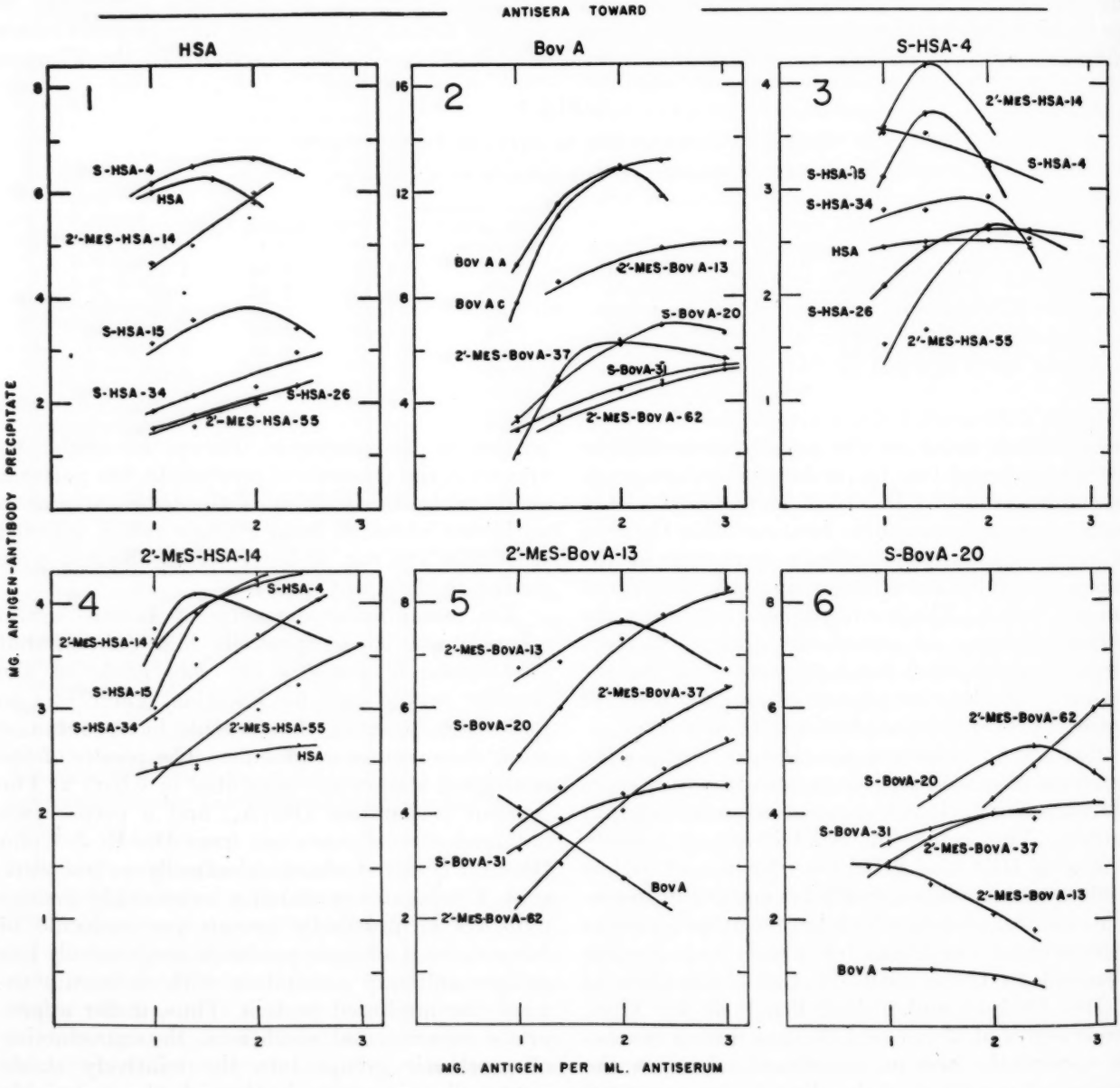
ANTISERUM	ANTIGEN	PER CENT DECREASE IN INTENSITY OF ABSORPTION	MG. ANTIGEN ADDED		
			1.0	1.4	2.0
2′-MeS-HSA-14	2′-MeS-BovA-37-D	0	1.07	1.16	1.16
2′-MeS-HSA-14	2′-MeS-BovA-37-L	27	1.25	1.42	1.62
2′-MeS-HSA-55	2′-MeS-BovA-37-D	0	0.49	0.46	0.49
2′-MeS-HSA-55	2′-MeS-BovA-37-L	27	0.55	0.62	0.55
2′-MeS-HSA-55	2′-MeS-BovA-62-D	0		1.47	1.54
2′-MeS-HSA-55	2′-MeS-BovA-62-L	27		1.63	1.67

Homologous protein series.—Chart 1 shows the reactions of pooled antiserum toward horse serum albumin with various test antigens containing that protein. The antiserum reacted almost equally well with HSA and S-HSA-4. Because of its low content of carcinogen, S-HSA-4 essentially represents control protein which had been subjected to experimental conditions of alkali and dioxane identical to those used for the preparation of 2′-MeS-HSA-14 and 2′-MeS-BovA-13 (3). Thus, the presence of 35 per cent dioxane during synthesis apparently had no significant effect on the serological reactivity of the albumin component of the test antigen. Conjugate 2′-MeS-HSA-14 gave less antigen-antibody precipitate at lower levels of antigen than S-HSA-4, presumably because of its higher content of prosthetic group. The serological reactions became increasingly weaker when conjugates containing greater numbers of prosthetic groups were added to this antiserum, as shown by the reactions with S-HSA-15, S-HSA-26, S-HSA-34, and 2′-MeS-HSA-55. Since the latter four conjugates were prepared in reaction media containing 44–48 per cent dioxane, it is again evident that the presence of increasingly greater numbers of groups per molecule of protein was predominantly responsible for the reduction in serological reactivity of the albumin component. Because of the difference between the reactions of 2′-MeS-HSA-14 and S-HSA-15, an increased percentage of dioxane during synthesis may have played a minor role in depressing the reactivity of the albumin portion of the conjugate. Except for slight decreases in the amounts of precipitate, the patterns produced by the addition of the same antigens to antiserum obtained from a single rabbit injected with only 180 mg. of horse serum albumin were similar to those in Chart 1.

The immunizing capacity of bovine serum albumin was so unexpectedly high (cf. 2), that electrophoretic analysis (9) was made of the Armour supply used for injection. There was no indication, however, that it could have contained more than a trace of globulin. The results of the serological studies are presented in Chart 2. The Armour preparation ($BovA_A$) and a preparation obtained several years ago from Dr. E. J. Cohn ($BovA_C$) behaved almost identically as test antigens. Conjugates containing increasingly greater numbers of prosthetic groups per molecule of bovine serum albumin produced progressively less antigen-antibody precipitate with antiserum toward the unaltered protein. Thus, under appropriate experimental conditions, the introduction of prosthetic groups into the relatively stable serum albumin molecule through the carbamido

linkage causes a gradual, but not complete, obliteration of the serological specificity of the protein molecule.

Charts 3–5 contain the results with antisera toward conjugates with low numbers of prosthetic groups. When tested with HSA, the antiserum toward S-HSA-4 (Chart 3) produced less than one-half the amount of precipitate observed in the reaction with antiserum toward HSA (Chart 1). The reactions of test antigens S-HSA-4 and 2′-MeS-HSA-14 were also less with antiserum toward S-HSA-4 than with antiserum toward HSA, but conjugates containing 26–55 prosthetic groups displayed approximately the same reactivity with both antisera. The introduction of four stilbenyl groups altered the immunizing capacity and specificity of the protein in a minor way which could be detected only by the use of unaltered protein or of conjugates containing a few prosthetic groups. Horse serum albumin reacted weakly with antiserum toward 2′-MeS-HSA-14 (Chart 4). Considered along with the rapidly rising levels of precipitate formed with the highly substituted proteins, this indicates that fourteen groups in the immunizing antigen were adequate for the production of recognizable prosthetic group activity. The results in Chart 5 substantiate this interpretation. Although 2′-MeS-BovA-13 was a stronger immunizing agent than S-HSA-4 and 2′-MeS-HSA-14, the introduction of similar numbers of groups had essentially the same effect on the antigenic properties of both proteins.

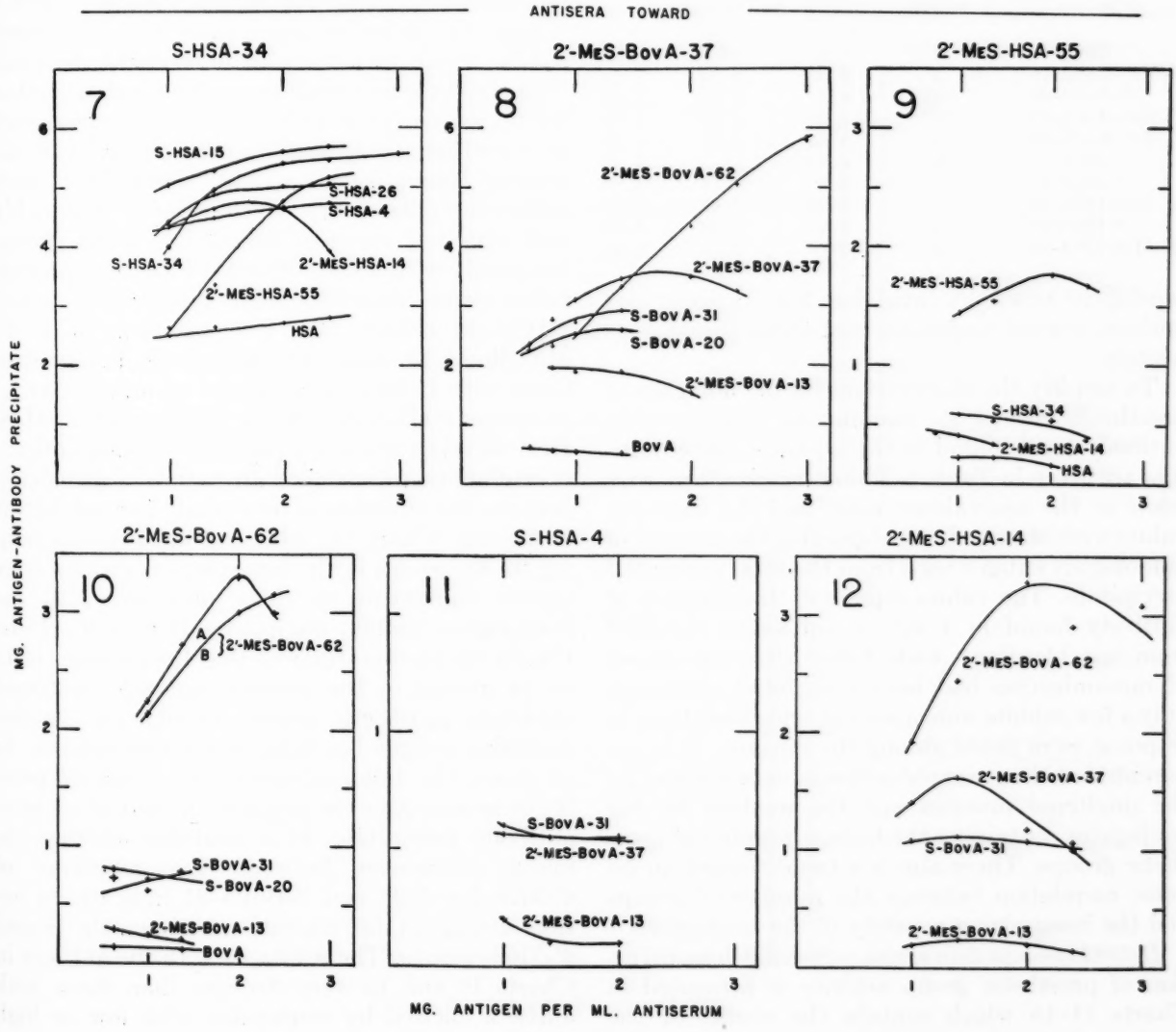


CHARTS 1–6

In all cases, the presence of a few prosthetic groups had a more pronounced influence on the immunizing antigen than on the test antigen. An increase in the number of prosthetic groups to twenty gave an antiserum (Chart 6) which reacted less strongly with test antigens BovA and 2′-MeS-BovA-13 in particular.

The effects of a moderate number (34–37) of prosthetic groups on the antigenic activity of the proteins are presented in Charts 7 and 8. From Chart 7 it will be seen that the introduction of 34 stilbenyl groups into horse serum albumin has modified the specificity considerably without appreciable loss of immunizing capacity. The most significant change from the previous findings was the increased amount of precipitate produced by the addition of 1–2 mg. of the test antigens containing higher numbers of prosthetic groups. When compared with Charts 2 and 5, the results with antiserum toward 2′-MeS-BovA-37 (Chart 8) show a reversal in the positions of the antigen-antibody curves of the antigens containing low and high numbers of prosthetic groups. At 1 mg of antigen/ml of antiserum, the homologous antigen 2′-MeS-BovA-37 produced the most precipitate, and the extents of reaction with the other antigens decreased in proportion to their content of prosthetic group. Much precipitate was produced by the addition of 2′-MeS-BovA-62, the least stable of the test antigens, to antisera toward S-BovA-20 and 2′-MeS-BovA-37. The results of serological studies with antisera toward S-HSA-26 and S-BovA-31 agreed with those described above.

The introduction of nearly the maximum possible number of 66 prosthetic groups had a pronounced effect on the immunizing capacity of the serum albumins. The homologous antigen-antibody reactions shown in Charts 9 and 10 were less than one-third those shown in Charts 1 and 2. The presence of 55 or 62 groups in the antigens also had



CHARTS 7–12

a striking influence on specificity in that the reactions between these antisera and their homologous antigens were about 3 times as extensive as those with any other test conjugate. The amounts of precipitate obtained with the unaltered proteins were extremely low. When present at high levels, it is evident that the prosthetic group played a dominant role in the immunological reactivity of the conjugate. When tested with antiserum toward 2′-MeS-BovA-62, the light-exposed antigen 2′-MeS-BovA-62_A was slightly more reactive than 2′-MeS-BovA-62_B, which had been kept in the dark until use.

Whether or not the 2′-methyl grouping was present in or absent from the 4-dimethylaminostilbenyl prosthetic groups was unimportant in the serological reactions involving test antigens and antisera toward conjugates containing homologous protein.

TABLE 3

ANTIBODY LEVELS AS DETERMINED BY HOMOLOGOUS TEST ANTIGENS

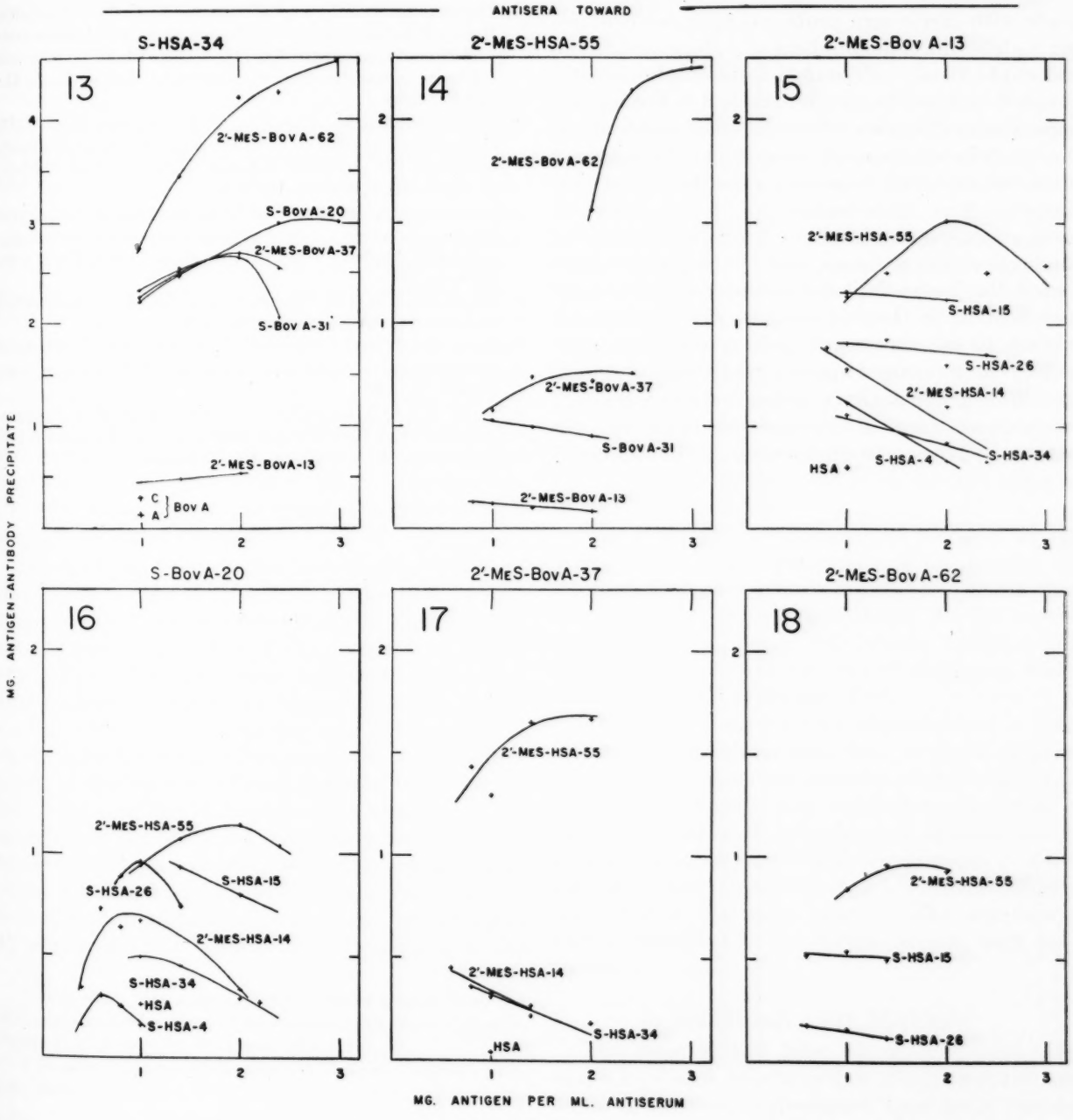
ANTISERUM	No. RABBITS	MG OF ANTIBODY/ML OF ANTISERUM		
		Lowest	Highest	Av.
BovA	4	4.95	12.6	9.51
2′-MeS-BovA-13	3	5.07	9.55	7.21
S-BovA-20	3	2.23	4.27	3.21
2′-MeS-BovA-37	2	2.60	3.00	2.80
2′-MeS-BovA-62	4	0.25	2.00	1.40
HSA	9	2.56	15.8	6.95
S-HSA-4	3	2.90	5.70	3.86
2′-MeS-HSA-14	3	2.69	4.92	3.82
S-HSA-34	3	2.06	7.48	3.90
2′-MeS-HSA-55	5	0.77	1.78	1.24

To amplify the observations on the influence of prosthetic groups on immunizing capacity, the antibody levels found in the separate rabbit antisera are given in Table 3. The determinations were made at the equivalence zone, and the antibody values were obtained by subtracting the amount of homologous antigen used from the total amount of precipitate. The values represent the amounts of antibody found in 1 ml. of antiserum obtained from test bleedings made 1 day after the course of immunization had been completed. Although only a few rabbits were used and wide variations in response were noted among the antisera, it is apparent that the strongest antisera were elicited by the unaltered proteins and the weakest by the conjugates containing the highest number of prosthetic groups. There also is a trend toward an inverse correlation between the number of groups and the immunizing capacity of the conjugates.

Heterologous protein series.—Specific demonstration of prosthetic group activity is presented in Charts 11–18 which contain the results of the serological reactions in which the protein components of the immunizing and test antigens were different. Neither horse serum albumin nor bovine serum albumin reacted with antisera toward the heterologous protein. The horse serum albumin conjugates also showed no signs of serological reaction when added to antisera toward bovine serum albumin. A trace of precipitate was produced by the addition of opalescent solutions of 2′-MeS-BovA-62 and S-BovA-20 to antisera toward horse serum albumin. The unaltered proteins reacted slightly with antisera toward some of the heterologous conjugates.

The amounts of antigen-antibody precipitate obtained upon testing the antisera with conjugates containing heterologous protein were appreciably less than those noted in the homologous series. In the former case, reaction can occur only when the prosthetic groups of the test antigen fit into the complementary regions of the antibody established by the stilbenyl groups of the heterologous immunizing protein. Antiserum toward S-HSA-4 (Chart 11) gave no precipitate with unaltered bovine serum albumin and only slight reactions with the three test conjugates. This indicates that the presence of four stilbenyl groups has conferred no more than a very weak haptenic activity on the protein. Immunization with 2′-MeS-HSA-14 gave antiserum (Chart 12) which reacted reasonably well with test antigens containing 31–62 groups but poorly with 2′-MeS-BovA-13. Greater precipitation was observed with antiserum toward S-HSA-34 (Chart 13). The reactions with 2′-MeS-BovA-13, however, were little better than those with Cohn bovine serum albumin ($BovA_C$) or Armour albumin ($BovA_A$). Although 2′-MeS-HSA-55 was a relatively poor immunizing antigen, it exhibited a pronounced alteration in specificity, because the amounts of precipitate formed by the antiserum (Chart 14) with test antigens containing 31–62 groups in the heterologous protein were almost equivalent to those obtained with the homologous protein conjugates (Chart 9). From Charts 12–14, it is apparent that the presence of 13 or 14 groups in the protein molecule conferred moderate prosthetic group activity on the immunizing antigen but little on the test antigen. In all cases, the test conjugate containing 62 prosthetic groups gave the greatest amount of antigen-antibody precipitate. It is doubtful whether the minor differences between the reactions of 2′-MeS-BovA-37 and S-BovA-31 indicate an actual serological differentiation between the S- and 2′-MeS- groups. The responses with the antisera in Charts 12 and 13 were stronger than those with antisera elicited by conjugates with low or high carcinogen content (Charts 11 and 14).

The results with antisera toward the bovine serum albumin conjugates are given in Charts 15–18. In every instance, the test antigen 2′-MeS-HSA-55 gave the strongest serological reaction. Although S-HSA-4 displayed little or no reactivity, 2′-MeS-HSA-14 was more reactive with antisera toward the heterologous protein conjugates containing 13–20 groups (Charts 15 and 16) than was 2′-MeS-BovA-13 (Chart 12). A further indication that conjugates prepared from the two proteins behaved differently as test antigens was the unexpectedly poor response of S-HSA-34 and the unusually good response of S-HSA-15 with antisera toward the bovine serum albumin conjugates. Although S-HSA-26 and S-HSA-34 would be expected to react about as well as S-HSA-15 and 2′-MeS-HSA-14 with antisera toward 2′-MeS-BovA-13 (Chart 15) and S-BovA-20 (Chart 16), it was surprising that the former test antigens gave so little precipitate with antisera toward the more highly substituted conjugates (Charts 17 and 18). Since the lysine side chains involved in the conjugation with the isocyanates probably are neither identically situated nor substituted in the two proteins, the presence of either a low number (15) or a high number (55) rather than a moderate



CHARTS 13–18

number (26–34) of prosthetic groups in the test antigen may permit, in certain cases, an over-all better fit and subsequent combination with the complementary regions of the antibody molecule.

Because of their satisfactory immunizing capacity and superior prosthetic group activity, conjugates containing 13–37 prosthetic groups should be the best preparations for experiments to determine whether protection against carcinogenesis due to 4-dimethylaminostilbene can be established by immunological means.

SUMMARY

Quantitative immunological studies have been made with carcinogen-protein conjugates containing widely different numbers of 4-dimethylaminostilbenyl- and 2′-methyl-4-dimethylaminostilbenyl-4′-carbamido groups attached to bovine and horse serum albumins. The serological reactivity of the protein component was found to decrease progressively with increased substitution of carcinogen. The introduction of four prosthetic groups conferred only slight haptenic activity on the immunizing antigens, and 13–14 groups represented the lower limit for demonstration of haptenic activity in the test antigens. Conjugates containing 13–37 carcinogen groups retained satisfactory immunizing capacity and displayed high prosthetic group activity as immunizing agents. A pronounced drop in immunizing capacity was caused by the introduction of 55–62 stilbenyl groups into the proteins. The influence of light in decreasing the intensity of absorption and in promoting *trans-cis* shifts in the stilbenyl prosthetic groups has been taken into consideration in the interpretation of the immunological results.

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Epidermal Carcinogenesis in the Mouse Induced by One, Two, or Three Applications of 9,10-Dimethyl-1,2-benzanthracene Followed by Repeated Applications of Croton Oil. A New Hypothesis of the Mechanism of Carcinogenesis*

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INTRODUCTION

Treating the skin of the mouse with a dose of 3,4-benzpyrene so small that, of itself, it is unable to make more than an occasional tumor appear, has been found to influence the skin so that subsequent applications of the noncarcinogenic substance croton oil are enabled to induce many tumors in the prepared area (1). Other carcinogens of the benzanthracene series act in the same way, and even a single application has been found adequate to prepare the skin (2, 8). These experiments provide the basis for the hypothesis that carcinogenesis in the skin of the mouse can be divided into two stages. In the first, the carcinogen prepares the skin so that, in the second, the noncarcinogenic agent croton oil, further applications of a carcinogen, or other adequate stimuli may make tumors manifest (3).

Some of the factors affecting the first stage of this process—the preparation of the skin by the carcinogen—have been investigated. When tumors are induced in the skin of the mouse by a single application of a carcinogen, followed by repeated applications of croton oil, there is a latent period during which no tumors are seen; then, a time during which more papillomas appear; and, finally, a period during which few if any new tumors arise (3). The number of tumors that croton oil can manifest in the prepared skin is thus limited. Once treated with carcinogen, the skin remains prepared indefinitely; for, whether the first application of croton oil be made a few days, or as long as 43 weeks after the application of carcinogen, the number of tumors made manifest by the oil is the same (5). The number of tumors induced depends on the nature and concentration of the carcinogen used for the preparatory application (3, 4). If different carcinogens are used for this initial application, croton oil manifests different numbers of tumors. If different concentrations of the same carcinogen are used to prepare the skin, the number of tumors croton oil can manifest varies with the concentration of carcinogen (4). It has also been noted that the average latent period of tumor induction remains constant whatever carcinogen is used for the preliminary application, and whatever its concentration is, provided the latent period be measured not from the time the carcinogen was applied but from the first application of croton oil (3).

When tumors are induced in the skin of the mouse by a single application of carcinogen followed by repeated applications of croton oil, they are at first all benign papillomas. As the experiment continues, many will remain benign papillomas, but some will regress and disappear, and a few will become malignant (10).

This paper reports two experiments: a pilot experiment in which one, two, or three applications of a 1.0 per cent solution of 9,10-dimethyl-1,2-benzanthracene was applied to the skin of mice and followed by repeated applications of croton oil, and a larger-scale experiment in which 0.2 per cent 9,10-dimethyl-1,2-benzanthracene was used in the same manner. The numbers of tumors manifest in the various groups were compared.

METHODS AND RESULTS

The mice in the pilot experiment were C3H males bred and maintained in this laboratory, and those in the main experiment were Swiss females bred at the Roscoe B. Jackson Memorial Labora-

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tory, Bar Harbor, Maine. All the mice were approximately 3 months old at the beginning of the experiments.

The carcinogen was either a 1.0 per cent solution or a 0.2 per cent solution of 9,10-dimethyl-1,2-benzanthracene (Eastman Kodak) in mineral oil (Superla 34, light mineral oil, Standard Oil of Indiana); the croton oil was a 5 per cent solution of Oleum crotonis B.P. (Boots) in the same mineral oil. An area 1.5 cm. square in the interscapular region was kept free of hair by clipping with scissors, and the solutions applied to this area with an eye dropper. Each dose of carcinogen was 1 drop and each dose of croton oil 2 drops.

The tumors were charted weekly for the first 30 weeks and thereafter every 2 weeks. Those tumors which regressed before they had been present 4 weeks have been disregarded. All others, whether persisting throughout the experiment or not, have been counted as tumors.

Experiment 1

Thirty male C3H mice were used in each of the three groups:

Group 1.—The mice were given a single application of 1.0 per cent 9,10-dimethyl-1,2-benzanthracene, and 5 weeks later croton oil was begun and was continued twice a week for 35 weeks.

TABLE 1

THE EFFECTS OF ONE, TWO, OR THREE APPLICATIONS OF CARCINOGEN FOLLOWED BY REPEATED CROTON OIL PAINTING

Group*	Survivors at time of first tumor	Av. latent period (weeks)	Tumor-bearing mice	Total no. tumors	Av. tumors/ tumor-bearing mice
1†	21	23.1	13	27	2.0
2‡	22	22.7	15	23	1.5
3§	16	20.2	13	24	1.8

* C3H male mice.

† A single application of 1.0 per cent 9,10-dimethyl-1,2-benzanthracene followed by croton oil.

‡ Two applications of 1.0 per cent 9,10-dimethyl-1,2-benzanthracene followed by croton oil.

§ Three applications of 1.0 per cent 9,10-dimethyl-1,2-benzanthracene followed by croton oil.

Group 2.—The mice received two applications of 1.0 per cent 9,10-dimethyl-1,2-benzanthracene with an interval of 1 week between applications. Five weeks after the first application, croton oil was begun and was continued twice a week, as in Group 1.

Group 3.—The mice were given three applications of 1.0 per cent 9,10-dimethyl-1,2-benzanthracene at weekly intervals. Five weeks after the first application, croton oil was begun and was continued twice a week as in Groups 1 and 2.

The results of this experiment are shown in Table 1. It can be seen that essentially the same number of mice bore tumors and the same numbers of tumors were induced irrespective of the number of applications of carcinogen administered. With a single application of carcinogen, thirteen tumor-bearing mice bore a total of 27 tumors; with two applications, fifteen mice had 23 tumors; and with three applications, thirteen mice had 24 tumors. The experiment was continued for 35 weeks from the first application of croton oil. No great differences were noted in the various latent periods.

Experiment 2

One-hundred female Swiss mice were used in each of the three groups:

Group 4.—The mice were given a single application of 0.2 per cent 9,10-dimethyl-1,2-benzanthracene, and 5 weeks later croton oil was begun and was continued twice a week for 36 weeks.

TABLE 2

THE EFFECTS OF ONE, TWO, OR THREE APPLICATIONS OF CARCINOGEN FOLLOWED BY REPEATED CROTON OIL PAINTING

Group*	Survivors at time of first tumor	Av. latent period (weeks)	Tumor-bearing mice	Total no. tumors	Av. tumors/ tumor-bearing mice
4†	87	10.9	53	416	7.8
5‡	88	11.8	59	305	5.1
6§	88	13.4	60	260	4.3

* Swiss female mice.

† A single application of 0.2 per cent 9,10-dimethyl-1,2-benzanthracene followed by croton oil.

‡ Two applications of 0.2 per cent 9,10-dimethyl-1,2-benzanthracene followed by croton oil.

§ Three applications of 0.2 per cent 9,10-dimethyl-1,2-benzanthracene followed by croton oil.

Group 5.—The mice received two applications of 0.2 per cent 9,10-dimethyl-1,2-benzanthracene, with an interval of 1 week between applications. Five weeks after the first application, croton oil was begun and was continued twice a week, as in Group 4

Group 6.—The mice were given three applications of 0.2 per cent, 9,10-dimethyl-1,2-benzanthracene at weekly intervals. Five weeks after the first application, croton oil was begun and was continued twice a week for 36 weeks, as in Groups 4 and 5.

The results are shown in Table 2: the more applications of carcinogen given, the fewer the tumors produced. With one application of carcinogen, 416 papillomas were made manifest, or 7.8 per tumor-bearing mouse; with two, only 305, or 5.2 per tumor-bearing mouse; and with three, 260, or 4.3 per tumor-bearing mouse. The average latent period measured from the first application

of croton oil was 10.9 weeks in Group 4, 11.8 weeks in Group 5, and 13.4 weeks in Group 6. Such differences in latent periods cannot be considered significant with the degree of variation seen.

It may be noted in passing that the results of this experiment only become striking when the total number of tumors is tabulated, the number of tumor-bearing animals being much the same in each group. This was also the case in previous experiments in which different concentrations of carcinogen were used (4). It would seem that in experiments of this sort it is essential to count the total number of tumors. Only in this way can quantitative comparisons be made.

DISCUSSION

One aspect of the results of these experiments is quite clear. Increased numbers of applications of a carcinogenic hydrocarbon within certain limits, when followed by applications of croton oil, will not increase the number of tumors induced. This result is seemingly at variance with the previously observed increase in the number of tumors seen with increasing concentrations of carcinogen followed by croton oil (4). In the first, and smaller, of these two experiments, similar numbers of tumors were noted in the various groups, the increases in numbers of applications having no apparent effect. However, in the larger of the two experiments, and that in which a lower concentration of carcinogen was used, a significant decrease in the number of tumors induced corresponding to increasing numbers of applications of carcinogen was observed. It should be noted that these two experiments cannot be compared in many details, as different strains of mice were used; the C3H mice appear considerably less responsive to this form of carcinogenesis than the Swiss mice.

Several explanations of the observed results seem possible within the limits of previous hypotheses of carcinogenesis. The view that skin carcinogenesis in the mouse, with a single application of a carcinogen followed by croton oil, represents the initial production of some "latent tumor cells" and the subsequent conversion of these to actual tumors by a different action has not been contradicted.

The difference between the effects of increasing concentrations or increasing numbers of applications of carcinogen requires additional explanation. In the first of these experiments it was noted that the same numbers of tumors were produced by one, two, or three applications of carcinogen followed by croton oil. In the second experiment, decreasing numbers of tumors were produced corresponding to increasing numbers of applications of carcinogen. There were several differences between the two experiments, particularly in the strains of mice, the concentrations of carcinogen used, and the relative numbers of tumors induced. It is possible that the significant decrease in the numbers of tumors noted in the second experiment, namely, 416, 305, and 260, respectively, was noted because of the large number of animals used and may have been missed in the first experiment. Nevertheless, hypothetical explanations of the observed phenomena would seem to apply equally well to the cases in which no increase was observed and the number of tumors induced remained constant, and those in which decreases were observed. Perhaps the well known necrotizing effect of the carcinogen (6, 9) or, possibly, some inhibitory action (7) accounted for these results. This does not appear a satisfactory explanation at the dosage levels used. Again, some kind of refractory state may be induced by the first application of carcinogen, making the tissue no longer susceptible to further change within the time intervals chosen. It is also possible that a combination of both necrotizing action and refractoriness accounts for the result of the second series of experiments.

However, the only conclusion that can be stated with some finality is that increases in the number of applications of a carcinogenic hydrocarbon within the present experimental limits, when followed by applications of croton oil, do not give rise to increasing numbers of tumors. This finding is a sharp contrast with that previously noted when increasing concentrations of carcinogen were followed by croton oil.

SUMMARY

1. C3H male mice received either one, two, or three skin applications of a 1.0 per cent solution of 9,10-dimethyl-1,2-benzanthracene followed by croton oil repeatedly. No differences in the tumor incidences were noted.

2. Swiss female mice received either one, two, or three skin applications of a 0.2 per cent solution of 9,10-dimethyl-1,2-benzanthracene followed by croton oil repeatedly. Decreasing numbers of tumors were recorded with increasing numbers of applications.

3. The possible significance of these findings is discussed. The secondary necrotizing action of carcinogenic hydrocarbons and the possible induction of a refractory state are considered as explanations.

ACKNOWLEDGMENTS

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The Heterologous Transplantation of Human Lung Cancer*

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In the routine heterologous transfer of unselected human tumors, approximately one-half of the growths are found to be in a dependent phase of development at the time of operation, and takes are not obtained (3). Analysis of the incidence of transplantability in relation to the primary site of the tumor shows a consistent variation with respect to several organs, and notable among these are the breast and the lung. At the time of first surgical approach, the majority of breast cancers are in a dependent phase and are not transplantable, while all the lung cancers studied have been autonomous, or transplantable. The present paper concerns the series of lung cancers. The results of transplantation are reported in relation to the fate of the patient, and corresponding data derived from the study of tumors of similar morphological type arising in other organs are presented for purposes of comparison.

MATERIALS AND METHODS

The anterior chamber and the brain of the guinea pig were used as transplantation sites in the primary transfers of the tumors. In a number of cases, corresponding sites in the mouse were also employed, and the transplantation reactions to several of the tumors carried serially were studied in this species as well as in the guinea pig. The technic of transfer has been described in detail (1, 2).

The tumors were derived from the operating room of the New Haven Hospital, and their transfer was undertaken as part of a general study of the heterotransplantability of human cancer. They do not represent a consecutive series of all the lung cancers removed during the period of study, and selection for transfer was made on a purely random basis. The tumor tissue, obtained from the primary lesion, involved lymph nodes or metastasis, was used immediately after operation or stored in sterile containers at ice-box temperature for short intervals of time. The material for transfer was selected for viability and cellular content after careful gross examination, and in many cases frozen sections were employed.

As a rule, each tumor was transferred to eight guinea pigs, but in the case of desmoplastic growths as many as twelve pigs were used. The selection of the eye or brain as a transplantation site was also based on the amount of desmoplasia, for in past experience it has been noted that the reaction to adult human connective tissue was less pronounced in the brain.

It is important in the present connection to emphasize the importance of care in the selection of fragments for transfer in heterologous transplantation experiments, particularly those involving human tumors. Human tumors, unlike mouse tumors, usually contain a large amount of desmoplastic connective tissue. This is an adult tissue and will not survive heterologous transfer. Furthermore, when present in quantity it appears to inhibit the growth of transplantable cancer cells, and, in a large series of heterologous experiments, it was found that the incidence of takes varied inversely with the proportion of desmoplastic connective tissue present in the tumor. Growth in laboratory animals is not associated with the desmoplasia characteristic of growth in man, and this factor can be neglected in subsequent animal passages.

The ability to select suitable fragments varies with the experience of the operator and determines the number of animals necessary to validate the experiment. In the present case, it has been found that, with the aid of frozen sections, at least one out of eight fragments selected from a highly scirrhous carcinoma contains a preponderance of parenchyma, and for this reason a minimum of eight animals are used in each test.

The point to be emphasized is that the incidence of takes in the first-generation passage of a heterologous tumor reflects its content of desmoplasia and the experience of the operator rather than any essential character of the neoplastic cells. From this point of view a single take is as expressive of the true nature of the tumor as a 100 per cent incidence, and, unless a complete uniformity of transplanted fragments with respect to desmoplasia and parenchyma is assured, a tabulation of the incidence of takes is not subject to meaningful interpretation. Accordingly, in the tables accompanying this paper the occurrence of takes following the transfer of a tumor is signified by a plus (+) sign and their nonoccurrence by a minus (−). In point of fact, the incidence of takes varied from one out of eight to eight out of eight, but this difference disappeared on second-generation transfer and thus denotes the action of an element present in the fragments obtained from the operative specimen but absent from those derived from the first-generation growth.

RESULTS

The results of heterologous transfer of the thirteen lung cancers in this series are shown in Table 1. It will be noted that all the tumors proved to be transplantable and that all the patients died within a few months of operation.

Morphologically, the tumors were all epidermoid in character: three were classified as pleomorphic in cell type, five as poorly differentiated, and five as anaplastic. The tissue used for transfer was obtained from the primary growth in three

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cases, from regional nodes in five, from distal nodes in four, and from a brain metastasis in one case.

Neither histological type nor derivation of the tissue used appeared to influence the behavior of the transplanted tumor. With the sole exception of Case No. 11, growth in the eye was apparent by the 10th day after transfer, and the anterior chamber was filled with tumor by the end of a month. The tumor in Case No. 11 showed the highest degree of differentiation in the series and grew slowly on transfer. Growth was first observed on the 8th day, and the chamber was not replaced until the 7th month. Serial transfer was not associated with an accelerated growth rate in this or in other cases.

The behavior of transplants in the brain was comparable to that of other tumors. Neurological signs were not apparent until the transplant had grown to occupy approximately half a cerebral hemisphere, and their appearance was rapidly followed by death of the animal. In general, death occurred in from 40 to 60 days after transfer, and different tumors were not associated with consistent variations in survival time.

Morphologically, the transplants were similar to the tissue used for transfer (Figs. 1–18). The degree of differentiation was comparable in the human tumor and the transplant, and, even in the case of the more anaplastic or "oat-cell" tumors, no indication of differentiation in the direction of an epidermoid structure was found. This is in contrast to experience with anaplastic tumors of other organs where transplants generally show a higher degree of differentiation and the possibility is suggested that the "oat-cell" tumor may be an entity in itself rather than an anaplastic epidermoid carcinoma.

The results obtained on the heterologous transfer of a series of epidermoid carcinomas derived from organs other than the lung is shown in Table 2. The tumors include three of the larynx, two of the hypopharynx, two of the skin, and one each of the lip, tongue, breast, cervix, urethra, and urinary bladder. The tissue used was obtained from a primary growth in one case, from regional nodes in eleven cases, and from a distant node in one.

Approximately one-half (six of thirteen) of these tumors proved to be heterotransplantable. This proportion is in general agreement with that obtained in the routine transfer of tumors derived from the operating room and in sharp contrast to the results described in the case of the lung.

TABLE 1

THE RESULTS OF THE HETEROLOGOUS TRANSFER OF A SERIES OF HUMAN LUNG CANCERS

Case no.	Patient	Tumor	Transfer			Present status of patient
			Date	Tissue used	Result	
1	V.B.(f)	Undifferentiated epidermoid carcinoma	4/21/48	Brain metastasis	+	Died 6/14/48
2	C.T.(m)	Anaplastic epidermoid carcinoma	9/28/48	Node chest wall	+	Died 10/10/48
3	B.K.(f)	Undifferentiated epidermoid carcinoma	12/16/48	Primary	+	Died 2/26/44
4	J.P.(m)	Anaplastic epidermoid carcinoma	5/ 3/48	Cervical node	+	Died 6/28/48
5	W.F.(m)	Anaplastic epidermoid carcinoma	12/20/48	Cervical node	+	Died 12/24/48
6	K.A.(m)	Pleomorphic epidermoid carcinoma	3/ 3/49	Mediastinal node	+	Died 7/ 3/49
7	J.R.(m)	Undifferentiated epidermoid carcinoma	3/15/49	Mediastinal node	+	Died 10/16/49
8	G.B.(m)	Anaplastic epidermoid carcinoma	5/19/49	Mediastinal node	+	Died 6/13/49
9	J.C.(m)	Undifferentiated epidermoid carcinoma	6/30/49	Mediastinal node	+	Died 4/ 2/50
10	C.M.(m)	Pleomorphic epidermoid carcinoma	1/ 5/50	Node chest wall	+	Died 1/18/50
11	L.L.(m)	Undifferentiated epidermoid carcinoma	6/27/50	Primary	+	Died 2/20/51
12	J.B.(m)	Pleomorphic epidermoid carcinoma	2/ 5/51	Primary	+	Died 4/25/51
13	S.O.(m)	Anaplastic epidermoid carcinoma	5/21/51	Mediastinal node	+	Died 8/ 7/51

DISCUSSION

Heterotransplantability does not characterize tumors from their inception but is an attribute attained late in the course of development and ap-

FIG. 1.—Anaplastic epidermoid carcinoma of lung from Case No. 2. ×275.

FIG. 2.—Guinea pig anterior chamber transplant of tumor shown in Figure 1. × 275.

FIG. 3.—Undifferentiated epidermoid carcinoma of lung from Case No. 3. ×275.

FIG. 4.—Guinea pig anterior chamber transplant of tumor shown in Figure 3. ×275.

FIG. 5.—Anaplastic epidermoid carcinoma of lung from Case No. 8. ×275.

FIG. 6.—Guinea pig anterior chamber transplant of tumor shown in Figure 5. ×275.

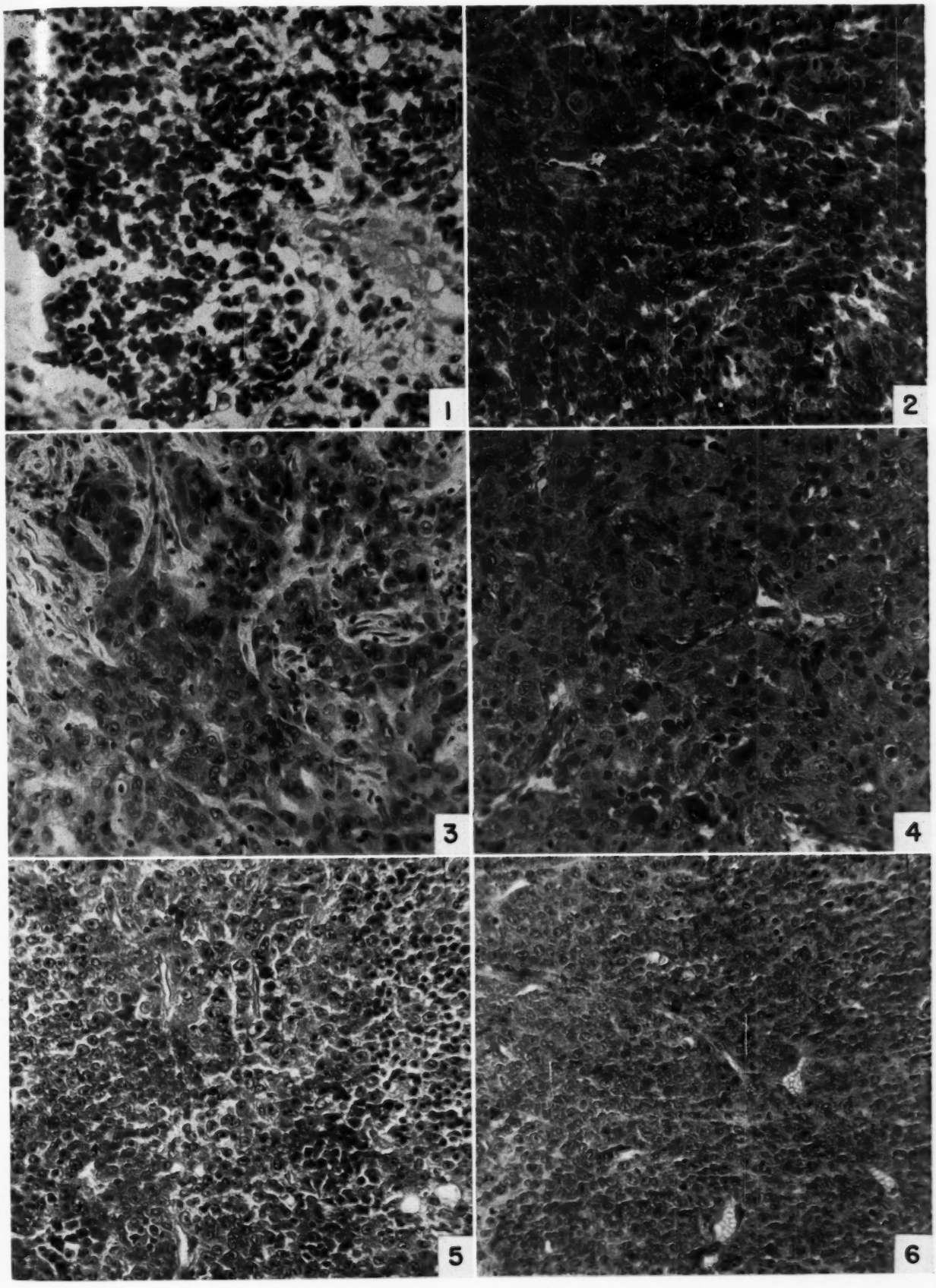
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FIG. 7.—Undifferentiated epidermoid carcinoma of lung from Case No. 9. ×275.

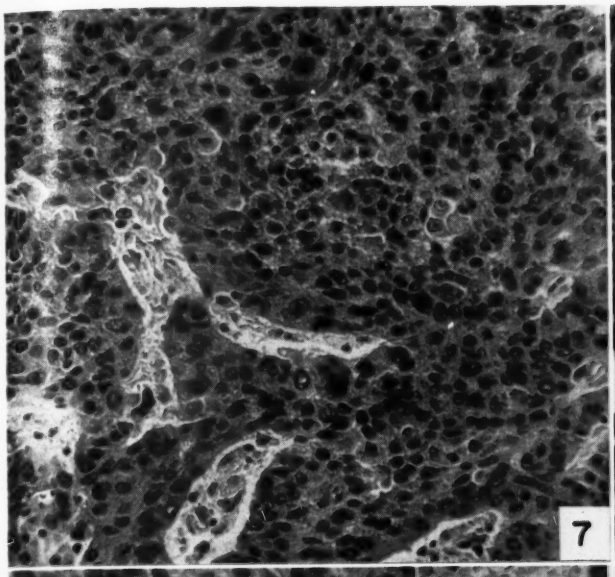
FIG. 8.—Mouse anterior chamber transplant of tumor shown in Figure 7. ×400.

FIG. 9.—Pleomorphic epidermoid carcinoma of lung from Case No. 10. ×275.

FIG. 10.—Guinea pig anterior chamber transplant of tumor shown in Figure 9. ×275.

FIG. 11.—Undifferentiated epidermoid carcinoma of lung from Case No. 11. ×275.

FIG. 12.—Guinea pig anterior chamber transplant of tumor shown in Figure 11. ×275.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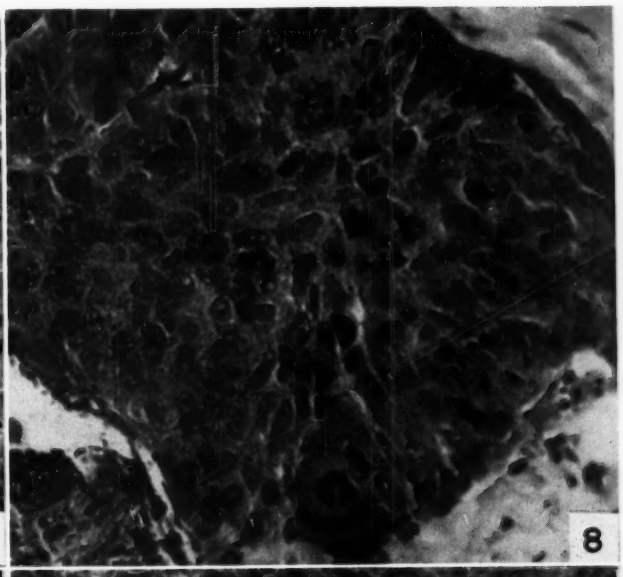
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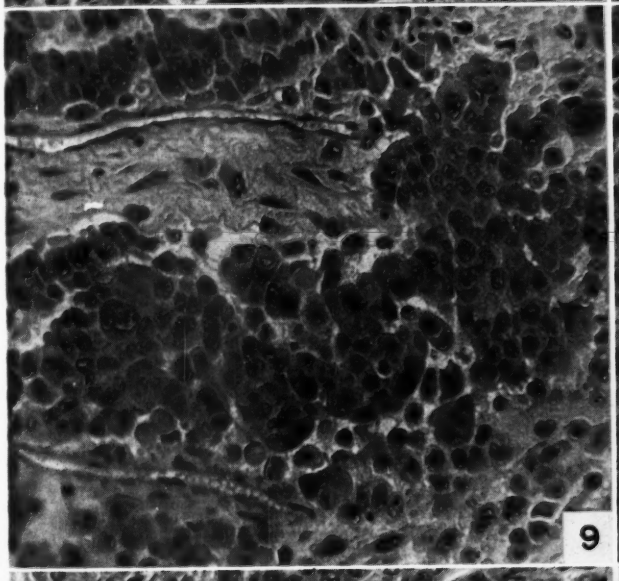
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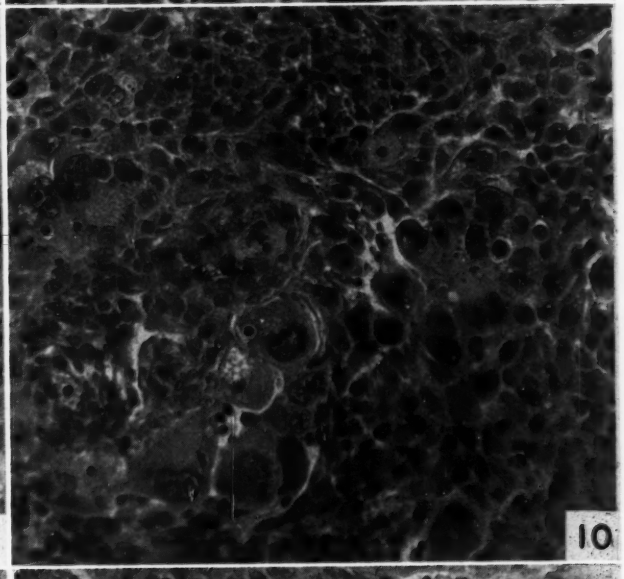
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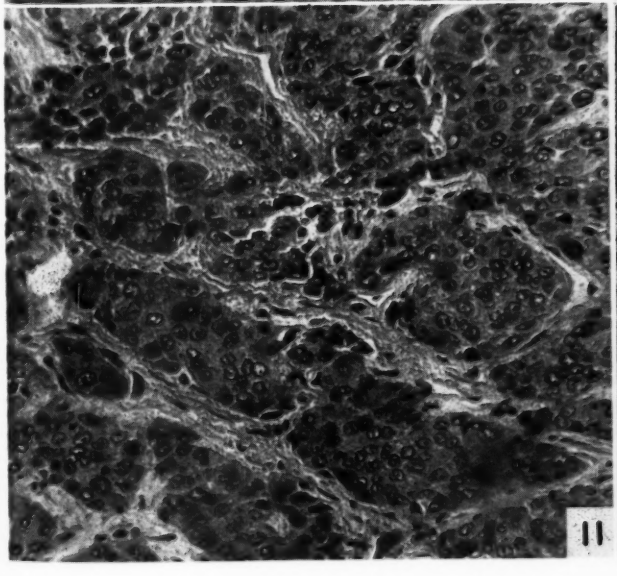
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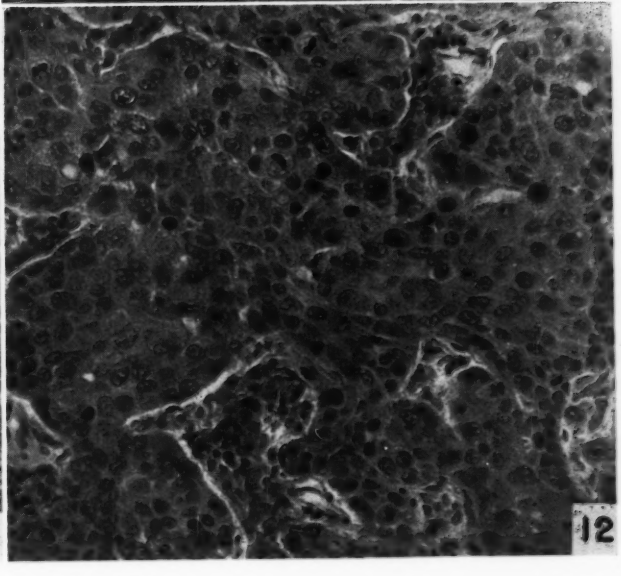
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FIG. 13.—Pleomorphic epidermoid carcinoma of lung from Case No. 12. ×275.

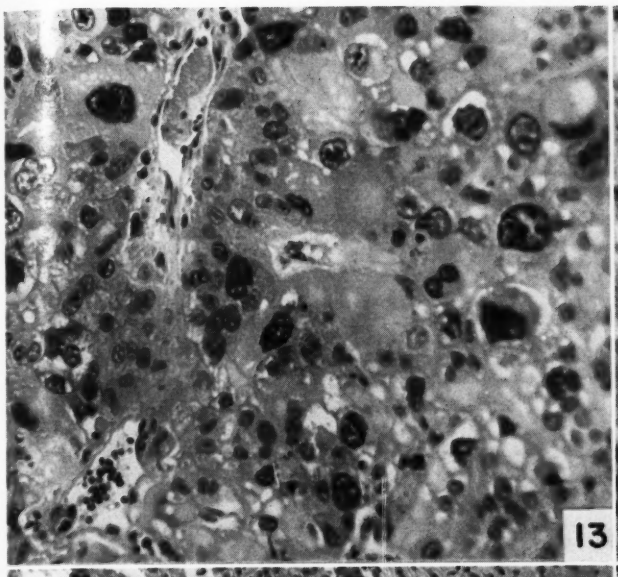
FIG. 14.—Guinea pig brain transplant of tumor shown in Figure 13. ×275.

FIG. 15.—Mouse brain transplant of tumor shown in Figure 13. ×135.

FIG. 16.—Anaplastic epidermoid (oat-cell) carcinoma of lung from Case No. 13. ×275.

FIG. 17.—Guinea pig brain transplant of tumor shown in Figure 16. ×275.

FIG. 18.—Mouse brain transplant of tumor shown in Figure 16. ×275.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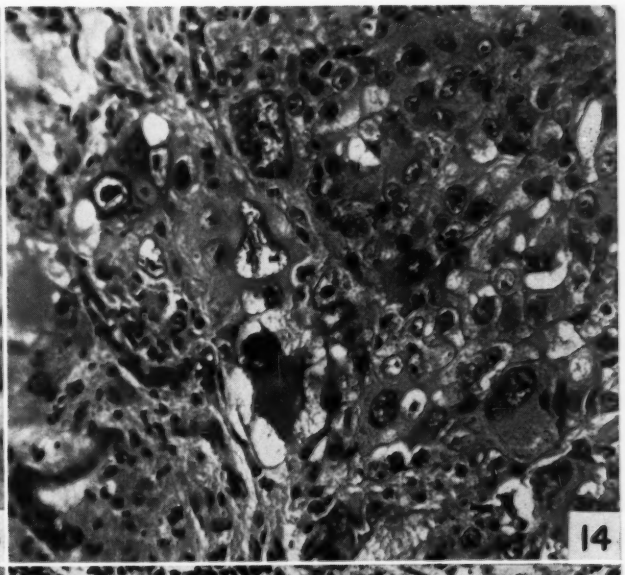
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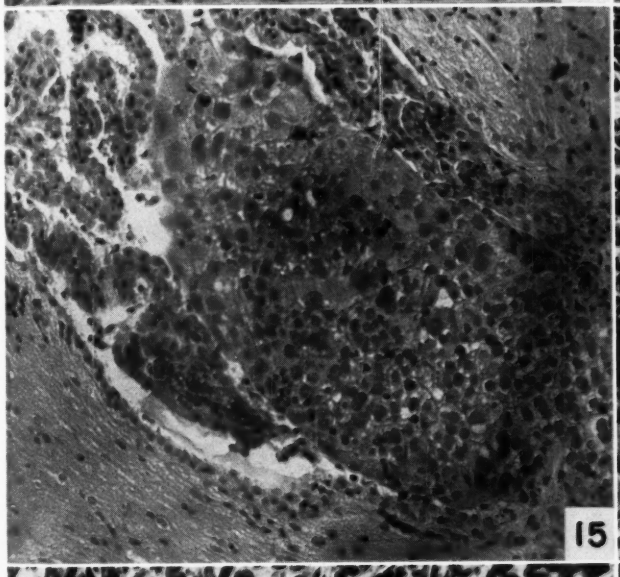
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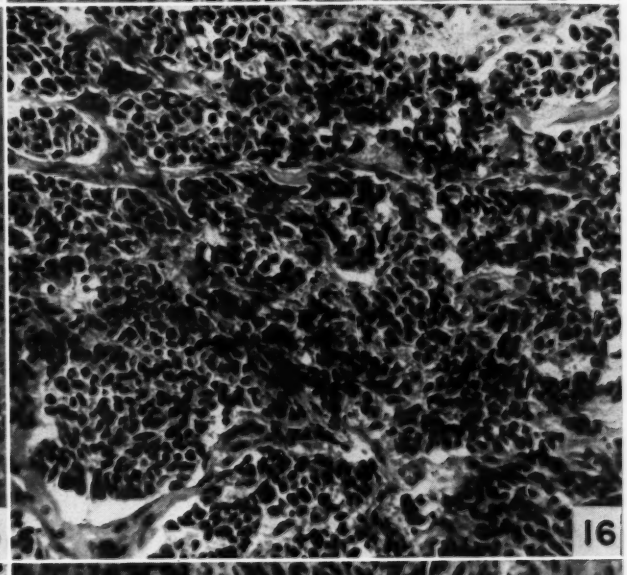
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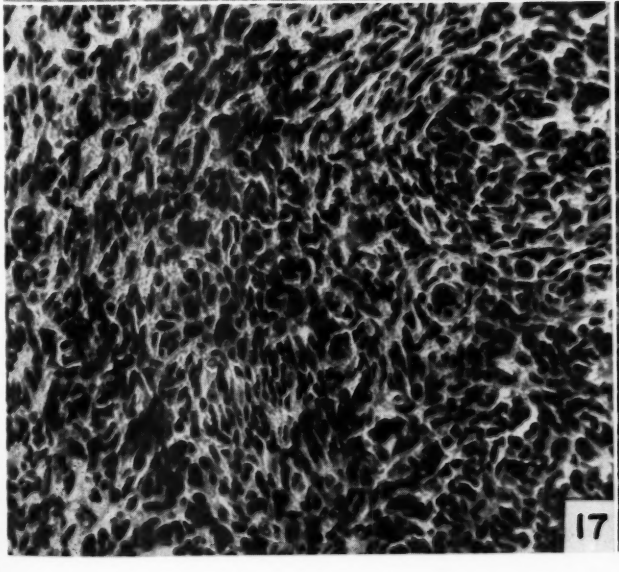
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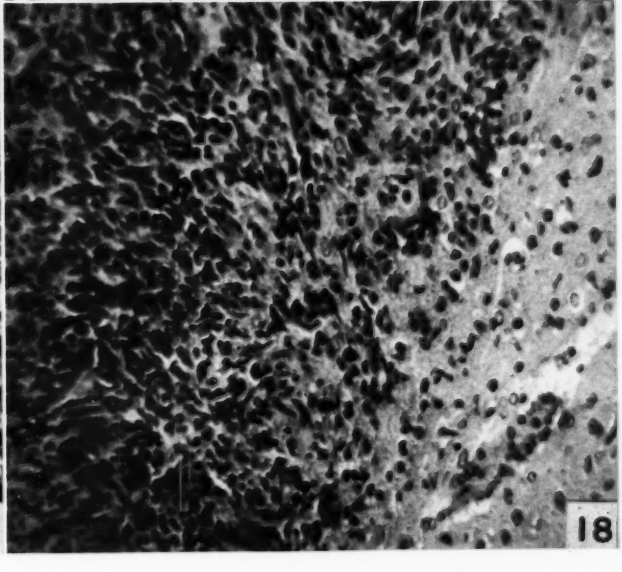
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pears to coincide with the attainment of the property of metastasizability (3). The results of the experiments reported in this paper represent the status of the tumors studied with respect to heterotransplantability and reflect their developmental state at the time of first surgical approach. In the great majority of cases this date closely approximates that of clinical discovery, and, in view of the disproportionate incidence of heterotransplantability in the two groups, it is apparent that, at this date, the tumors of the lung had reached a more advanced stage of development than those of other organs. This finding is of clinical significance and may be interpreted as meaning either that the developmental advance from dependency to autonomy is more rapid in lung tumors than in tumors of other organs or that the developing neoplasm is present in the lung for a longer period of time before clinical discovery.

TABLE 2

THE RESULTS OF THE HETEROLOGOUS TRANSFER OF A SERIES OF EPIDERMOID CARCINOMAS DERIVED FROM ORGANS OTHER THAN THE LUNG

Case no.	Patient	Primary site	Transfer			Present status of patient
			Date	Tissue used	Result	
1	R.S.(f)	Tongue	3/31/43	Cervical node	+	Died 7/20/43
2	C.P.(m)	Urethra	5/16/44	Inguinal node	+	Died 9/ 1/45
3	L.A.(m)	Hypopharynx	11/ 5/47	Cervical node	−	Alive
4	D.P.(m)	Skin	1/ 8/48	Primary	−	Alive
5	E.L.(m)	Hypopharynx	10/13/48	Cervical node	+	Died 2/21/49
6	D.B.(m)	Larynx	11/30/49	Cerivcal node	−	Died 2/21/51
7	D.D.(m)	Larynx	2/ 9/50	Cervical node	−	Died 8/25/50
8	G.C.(m)	Breast	7/ 5/50	Axillary node	+	Lost to follow-up
9	D.C.(m)	Lip	7/21/50	Cervical node	−	Died 12/ 4/50
10	L.W.(f)	Cervix	7/28/50	Inguinal node	−	Alive
11	R.P.(m)	Bladder	8/ 3/50	Cervical node	+	Died 7/14/51
12	K.H.(f)	Larynx	10/ 6/50	Cervical node	+	Died 12/21/50
13	M.D.(f)	Skin	10/12/50	Axillary node	−	Alive

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Cytological Studies on the Ehrlich Ascites Tumor before and after Infection with Bunyamwera Virus*

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The effect of virus infection upon transplantable mouse tumors has been the subject of several investigations in the past (12, 13, 26). More recently, studies have been published on the inhibitory action of Russian Spring-Summer encephalitis virus (18, 19) and of West Nile, Bunyamwera, and Ilheus viruses (9) on the growth of a variety of transplantable mouse tumors. Inhibition of tumor growth has been demonstrated by the measurement of solid tumors and the results of bioassay, but only a very few histopathological observations have been made. Koprowska and Koprowski (8) placed this work on a more quantitative basis by utilizing the ascites tumors (7). The availability of tumor cells in a fluid suspension made possible a numerical assessment of the amount of destruction produced by the virus, and the results of subsequent transplantation of the tumors were not vitiated by the occurrence of nonspecific necrosis in the controls. In the course of their observations on the effect of twelve neurotropic viruses upon the Ehrlich ascites tumor, Koprowska and Koprowski (8) repeatedly observed the oncolytic action of Bunyamwera virus on the tumor. They noted that tumor cells were absent from the peritoneal exudate 4–5 days after intraperitoneal inoculation of the virus, and attempts at transplantation into immune mice failed to elicit the growth of tumors. The present experiments are an extension of this study and were carried out in order to elucidate and observe chronologically the changes in the Ehrlich ascites tumor produced by infection with Bunyamwera virus. The work was designed to investigate the cytochemical and morphological characteristics of the tumor before and after virus inoculation, with a view to interpreting the pathological changes in relation to the "physiology" of the uninfected tumor cell.

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MATERIALS AND METHODS

Biological Procedures

Mice.—Swiss albino mice were used for virus titrations and Strong A or Swiss albino mice for all other work. The mice weighed 18–22 gm. and were supplied by commercial dealers.

Virus.—Bunyamwera virus (25) was obtained through the courtesy of Dr. K. Smithburn of the Division of Medicine and Public Health of the Rockefeller Foundation. The virus was kept frozen in the form of a 20 per cent suspension in saline of Bunyamwera-infected mouse brains. The final virus inoculum was prepared from this material; it consisted of 0.25 cc. of a 10 per cent suspension in saline of infected mouse brain, and represented the 35th–40th mouse brain passage of the virus since its original isolation (25).

Tumor.—The Ehrlich ascites tumor (7) was kindly sent to us by Dr. George Klein of the Karolinska Institute in Stockholm.

The tumor inoculum consisted of 0.2 cc. of undiluted pooled ascitic fluid withdrawn from mice on the seventh to ninth days of tumor growth and contained not less than 10 million tumor cells.

In each experiment the tumor was inoculated intraperitoneally into sixteen mice. Four days later, one-half of the mice were given intraperitoneal inoculations of virus, and the other half of a 10 per cent suspension of normal mouse brain—to serve as controls. During the next few days, individual mice in each group were tapped at frequent intervals, and the fluid prepared for cytological examination. In order to avoid errors due to trauma and repeated withdrawal of fluid, different mice were tapped each time. Three days after virus inoculation (7 days after implantation of the tumor), all the control and virus-infected mice were tapped and the pooled ascitic fluid was used for virus titration and bioassay.

Virus titration.—For the purpose of virus titration, pooled ascitic fluid was centrifuged for 10 minutes at 1,500 r.p.m. Serial tenfold dilutions of the supernatant were prepared in 10 per cent normal rabbit serum in saline and injected intracerebrally into mice. Six mice per dilution were used. In some instances, supernatant obtained by centrifugation of uninfected ascitic fluid was also injected intracerebrally into mice to confirm the fact that deaths of mice injected with virus-infected fluids were caused by virus and not by the presence of tumor cells in the fluid.

Bioassay.—Bioassay was carried out by subcutaneous inoculation into groups of six to eight mice of samples of 0.2 cc. of the remainder of the uncentrifuged pooled ascitic fluid from the virus-infected and control mice. Four experiments in all were done; in three of the four experiments, viz. nos. 1, 3, and 4, the mice had been immunized against Bunyamwera virus. The results were determined by the development of progressive solid tumors at the site of inoculation.

CYTOLOGICAL PROCEDURES

Smears of the ascitic fluid were fixed wet in formol sublimate, and the following procedures were carried out:

a) Barrett's bone marrow stain (3) before and after treatment with 0.1 per cent ribonuclease (Worthington Biochemicals) (20); (*b*) the periodic acid Schiff (PAS) technic of McManus (17); (*c*) Danielli's tetrazotized benzidine reaction before and after benzoylation with 10 per cent benzoyl chloride in dry pyridine for 20 hours at room temperature (4); (*d*) Lendrum's phloxin tartrazine stain (11); (*e*) staining with 1 per cent aqueous toluidine blue, before and after treatment with ribonuclease (20). The efficacy of the ribonuclease preparation was confirmed by testing its ability to reduce the cytoplasmic basophilia of plasma cells. In the PAS method, the omission of the periodic acid in control sections gave a negative result.

Smears were fixed wet in Baker's formol calcium (1) and the following technics were used:

a) Baker's Sudan black B stain (1) before and after boiling the smears for 10 hours in equal parts chloroform and methanol; (*b*) the PAS technic (17); (*c*) the Schultz cholesterol method (14); (*d*) Lorrain Smith's Nile blue sulphate stain (16); (*e*) Baker's modification of the Smith-Dietrich method (2); (*f*) the performic acid Schiff technic (15). Smears were brominated for 1 hour and acetylated for 24 hours according to the method described by Lillie (15).

Smears were fixed wet in Heidenhain's Susa and stained with a mixture of aniline blue and orange G at a pH of 2.5, according to the method of White (28). Gardikas and Israels' modification of the Feulgen method (5) was used, except that the smears were fixed while wet; the fixative consisted of: methyl alcohol, 15 parts; 5 per cent acetic acid, 5 parts; formalin, 1 part; water, 5 parts. Smears were fixed in Regaud's fluid and stained with iron hematoxylin for mitochondria (22). Supravital staining was done with neutral red and Janus green (27). Dry films were stained with Leishman's blood stain.

Suspensions of cells were examined by phase microscopy and for birefringence with the polarizing microscope.

In every case where histochemical tests were negative, the method was checked by simultaneously testing a known positive substance.

Four hundred cells were counted for the main differential and 500 tumor cells for mitotic figures and degenerate cells. Definite nuclear disintegration was the criterion of degeneration. Following the standards used by Glucksmann (6), only the grosser types of mitotic abnormality were classified as abnormal mitoses. Bridge formation and multipolar divisions were not regarded as abnormal for the purposes of this count, since they do not necessarily lead to the death of the daughter cells. Control and virus-infected mice were tapped simultaneously to avoid any artificial differences which might be due to diurnal variations in mitotic activity.

RESULTS

Cytological observations on the uninfected Ehrlich ascites tumor cell.—The tinctorial and histochemical properties of the uninfected tumor cells are presented in Table 1. The appearance of smears stained with Leishman and Barrett's stains is shown in Figures 1 and 2. The periphery of the basophilic cytoplasm contains a number of unstained vacuoles which, in smears fixed in formol-calcium, contain sudanophilic material; on analysis by the method of Lison (16), this appears to consist of unsaturated neutral fat (Table 1). According to Lison, in the absence of cholesterol, cholesterides, and substances giving a positive Smith-Dietrich reaction, a colorless anisotropic sudanophilic substance which gives a pink reaction with Nile blue sulphate is an unsaturated neutral fat. Corroborative evidence is provided by the negative PAS reaction and the ready solubility in fat solvents.

In supravital preparations a number of small granules in the perinuclear cytoplasm stain with neutral red: similar granules are strongly sudanophilic and PAS-positive in formol calcium-fixed smears and presumably correspond to the lipochondria of Ries (23). Of the colorless lipids, glycolipids, some phospholipids, and some unsaturated lipids give a positive PAS reaction (30). The negative Smith-Dietrich reaction eliminates phospholipids, and the poor solubility in fat solvents suggests that the lipochondria do not contain neutral fat or fatty acids. The performic acid Schiff reaction for ethylenic groups (15) is negative, and the absence of these groups is confirmed by the fact that previous bromination does not block the positive periodic acid Schiff reaction; on the contrary, acetylation completely abolishes it. The reactive group is therefore a 1,2-glycol or a hydroxy-amino linkage. On the basis of elimination of other lipoid substances, the reactive groups are probably present in a glycolipid.

Numerous small, rod-shaped mitochondria scattered throughout the cytoplasm stain supravitally with Janus green and, in fixed smears, with Heidenhain's iron hematoxylin.

In cells stained by Barrett's method (Fig. 2) the nucleus has a fine membrane and contains a variable number of red nucleoli (on the average, four) each surrounded by a chromatin membrane. A few small threads or granules of chromatin are scattered throughout the nucleus. In addition, the nucleus contains many minute red granules of parachromatin and a pink background of structureless material or nuclear sap. The staining reactions of the latter are difficult to assess against the cytoplasmic background which is inevitably present. With Barrett's stain, however, the distinct contrast between the pink staining nuclear sap and the blue cytoplasm suggests that the acidophilic material is intranuclear. Since, according to Pollister (21), the affinity of cellular material for acid dyes indicates a high protein content, it seems probable that the nuclear sap consists of a dilute solution of protein.

In wet-fixed and in fresh preparations examined with the phase microscope the configuration of the nucleus is sometimes extremely irregular (Fig. 2). Multiple pseudopodia, each containing one or

TABLE 1

HISTOCHEMICAL AND TINCTORIAL PROPERTIES OF THE EHRLICH ASCITES TUMOR CELL

Cell Component	Barrett's stain	Leishman stain	Feulgen	PAS	Sudan B	Smith Dietrich	Acid Aniline Blue Orange G	Tetrazonium reaction	Tetrazonium after benzoylation	Nile blue	Schultz cholesterol	Supravital	Phloxine tartrazine	Toluid. blue	Toluid. blue after ribonuclease	Birefringence	Solubility in fat solvents	Conclusion
Chromatin	H	Purple	+	0	0	0	Blue	++	+	0	0	0	H	Blue	Blue	0	Insoluble	Desoxyribonucleic acid protein
Nuclear sap	Pink	NR	0	0	0	0	Blue	+	0	0	0	0	Yellow	Pale blue	Very pale blue	0	Insoluble	Protein
Parachromatin granules	Red	NR	0	0	0	0	Orange yellow	++	0	0	0	0	Red	0	0	0	Insoluble	Histone
Nucleoli	Red	NR	0*	0	0	0	Orange yellow	++	0*	0	0	0	Red	0*	0*	0	Insoluble	Histone
Pseudonucleoli	Red	NR	0	0	0	0	Orange yellow	++	0	0	0	0	Red	0	0	0	Insoluble	Histone
Cytoplasm	Blue†	Blue	0	0	0	0	Blue†	++†	+	0	0	0	Yellow†	Blue†	Pale blue†	0	Insoluble	Ribonucleic acid protein
Lipochondria	0	0	0	+	+	Pale grey	0	0	0	Blue	0	Neutral red	0	0	0	0	Relatively‡ insoluble	Lipid (?) glycolipid
Cystoplasmic globules	0	0	0	0	+	0	0	0	0	Pink	0	0	0	0	0	0	Relatively§ soluble	Unsaturated neutral fat
Mitochondria	0	0	0	0	0	0	0	0	0	0	0	Janus green	0	0	0	0	Not tested	

O = Negative or unstained.
H = Hematoxyphil.
NR = Not recognizable due to insufficient detail.

* Only the nucleolar membrane stained.
† Often paler staining in nuclear "hof."
‡ Not removed by 10 hours' boiling in chloroform-methanol after formalin fixation.

§ Removed by methyl alcohol or xylol.

TABLE 2

DETAILED RESULTS OF EXPERIMENT 1

	Day 2																Day 3													
Time	9 A.M.		11 A.M.		1 P.M.		3 P.M.		5 P.M.		7 P.M.		9 P.M.		11 P.M.		2 A.M.		4 A.M.		6 A.M.		8 A.M.		10 A.M.		12 NOON		2 P.M.		4 P.M.	
	B	C	B	C	B	C	B	C	B	C	B	C	B	C	B	C	B	C	B	C	B	C	B	C	B	C	B	C	B	C	B	C
Tumor cells (per cent of total cells)	70	61	79	72	32	83	76	73	81	77	56	77	78	39	30	83	35	48	3	70	57	78	2	74	1	66	48	79	0	42	37	60
Degenerating tumor cells (per cent of tumor cells)*	10	0	†	0	7	†	2	0	†	0	1	0	2	†	26	†	28	0	42	†	15	0	75	0	40	0	31	†	0	†	33	†
Mitosis (per cent of tumor cells)	1.4	5.0	4.6	8.0	3.2	7.6	5.6	6.2	5.2	7.0	3.0	4.0	4.6	3.6	0	4.8	0	5.2	0	6.0	2.4	5.8	0	2.4	0	3.8	0	7.4	0	4.2	1.2	5.0
Percentage of mitosis in Prophase	0	8	9	13	6	21	4	10	8	6	7	5	9	6	0	17	0	38	0	10	8	0	0	17	0	16	0	14	0	24	16	20
Metaphase	29	16	26	25	19	24	21	29	20	20	20	35	30	16	0	12	0	23	0	20	42	10	0	17	0	47	0	24	0	14	17	16
Anaphase	14	8	0	0	6	8	4	10	11	6	13	5	4	6	0	0	0	8	0	7	0	7	0	8	0	0	0	5	0	0	0	4
Telophase	57	68	65	62	69	47	71	51	61	68	60	55	57	72	0	71	0	31	0	63	50	83	0	58	0	37	0	57	0	62	67	60
Abnormal mitosis (per cent of total mitosis)	0	0	4	3	6	3	7	0	0	0	27	0	13	6	0	0	0	0	0	0	8	14	0	25	0	5	0	5	0	0	0	4

	Day 4		Day 5		Day 7		Mean of observations		Difference between means	Standard error of difference between means
Time	4 P.M.		10 A.M.		10 A.M.					
	B	C	B	C	B	C	B	C		
Tumor cells (per cent of total cells)	1	43	0	59	0	39				
Degenerating tumor cells (cent of tumor cells)*	33	†	0	†	0	0				
Mitosis (per cent of tumor cells)	0	5.8	0	4.0	0	4.2				
Percentage of mitosis in Prophase	0	14	0	15	0	14	7.3	14.1	6.8	2.347
Metaphase	0	21	0	25	0	29	24.9	22.3	2.6	3.579
Anaphase	0	3	0	5	0	0	5.8	4.7	1.1	2.939
Telophase	0	62	0	55	0	57	61.9	58.9	3.0	3.425
Abnormal mitosis (per cent of total mitosis)	0	3	0	0	0	0	7.2	3.6	3.6	3.062

* 500 tumor cells counted where possible.
† Less than 1 per cent.

B = Bunyamwera infected group
C = Control.

more nucleoli, protrude from the surface. In a few cells the pseudopodia are extruded into the cytoplasm, and a form of nuclear budding or amitotic division results in the formation of a multinucleated cell (Fig. 3A): there is no evidence of prophase formation, and chromosomes cannot be visualized during the process. In less than 1 per cent of the tumor cells the buds are so numerous that complete distintegration of the nucleus results (Fig. 3B).

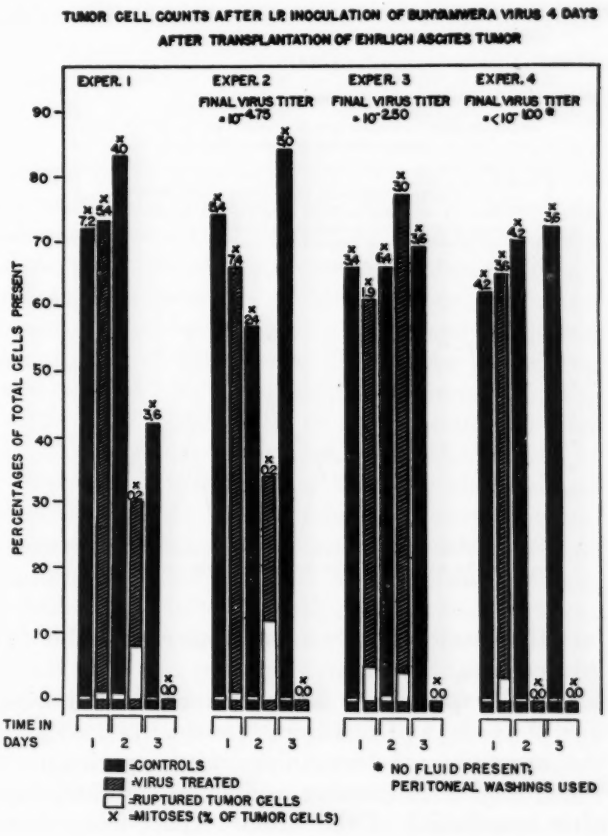
In a very rare cell (about one in 500 tumor cells) the nuclear sap is colorless, and the fine chromatin and dustlike parachromatin granules are absent from part or the whole of the nucleus. The chromatin is precipitated onto the nuclear membrane and around the nucleoli. The clear spaces left in the nucleus contain one or more round acidophil bodies. All gradations can be observed from the rare nuclei containing one or two acidophil bodies to the most common type with dustlike parachromatin granules.

Histochemical technics (Table 1) have failed to show any difference in the properties of the parachromatin, "acidophil bodies," and the nucleoli, except that the last have a chromatin membrane. All stain strongly with orange G and erythrosin and are therefore acidophil and rich in protein (10, 21). They have a stronger affinity for acid solutions of orange G than any other cell components which fact, according to White (28), implies a high content of histone. It was not possible to demonstrate ribonucleic acid in the nucleoli—or in the parachromatin and pseudonucleoli. These structures do not stain with toluidine blue, and, as might be expected, their affinity for the acid dyes of Barrett's stain is unaffected by ribonuclease digestion. Benzoylation eliminates the reaction of tyrosine, tryptophan and histidine with tetrazotized benzidine but has little effect on the reactivity of nucleic acids (4). The deep red coloration of the nucleoli with this reagent is completely abolished by benzoylation. The amount of ribonucleic acid, if present, is therefore insufficient to react with the tetrazo compound.

The "acidophil bodies" are not attached to chromosomes, and, like nucleoli and the parachromatin granules, they disappear in late prophase and reappear in late telephase. In the process of amitotic nuclear division described above they occasionally accompany and, in the smaller nuclear pseudopodia, even replace the nucleoli. The "acidophil bodies" would therefore appear to be derived from parachromatin granules, and, except for the absence of a chromatin membrane and chromosomal attachment, they are chemically and functionally similar to nucleoli. They might therefore be called parachromatin bodies or pseudonucleoli.

The effects of Bunyamwera virus on the Ehrlich ascites tumor.—In the first three experiments with Strong A mice (Chart 1), the percentage of tumor cells in the virus-infected fluids dropped on the second or third day. Similarly, the percentage of mitotic figures in the surviving cells fell rapidly. Parallel with this, the percentage of tumor cells showing nuclear disintegration increased to as much as 12 per cent of the total cells present, as compared to the percentage in the controls, which never rose above 1 per cent. By the third day, in all instances, only a rare tumor cell could be detected in the smears, and the exudate consisted of inflammatory cells, among which large macrophages and lymphocytes were predominant. Within the next 3 days, the fluid was completely absorbed.

CHART 1

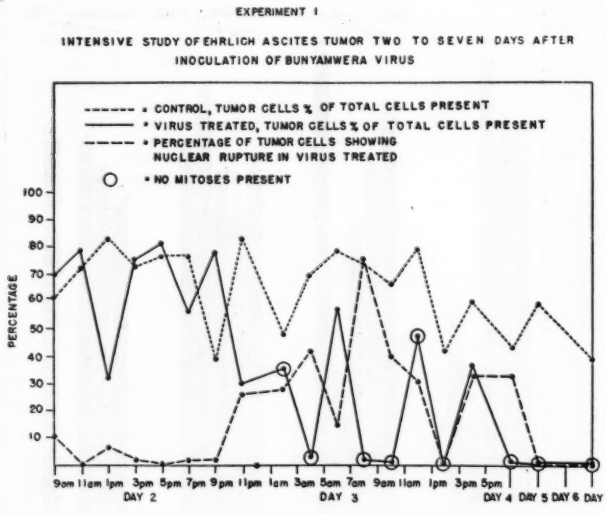

TUMOR CELL COUNTS AFTER I.P. INOCULATION OF BUNYAMWERA VIRUS 4 DAYS AFTER TRANSPLANTATION OF EHRLICH ASCITES TUMOR
EXPER. 1
EXPER. 2
FINAL VIRUS TITER = 10^4.75
EXPER. 3
FINAL VIRUS TITER = 10^2.50
EXPER. 4
FINAL VIRUS TITER = <10^1.00 *
PERCENTAGES OF TOTAL CELLS PRESENT
TIME IN DAYS
=CONTROLS
=VIRUS TREATED
=RUPTURED TUMOR CELLS
X =MITOSES (% OF TUMOR CELLS)
* NO FLUID PRESENT; PERITONEAL WASHINGS USED

On the third day after virus inoculation, when the fluid was tapped for bioassay and virus titration, the following results were observed. None of the mice injected subcutaneously with the fluids obtained from virus-infected animals developed tumors. On the other hand, tumors developed in all the animals which received fluids from uninfected controls and attained considerable size be-

fore any of the other group of mice died from virus infection. The titer of virus in the ascitic fluid was $10^{-4.75}$ and $10^{-2.50}$ in experiments 2 and 3, indicating that virus had multiplied in the tumor.

Experiment 4, with Swiss mice, was similar to the others except that tumor cells had disappeared from the fluid by the second day. As no fluid was available on the third day, saline washings of the peritoneum were used for virus titration and bioassay. Tumors failed to develop in mice inoculated subcutaneously with this material, and the virus titer was less than $10^{-1.00}$. Failure to demonstrate presence of virus was probably due to the

CHART 2



fact that ascitic fluid was no longer available for intracerebral titration.

In all experiments Bunyamwera-treated mice died 9–16 days after tumor transplantation, with the exception of two animals in experiment 4 which died with massive ascites on the 30th day after inoculation of the tumor. Apart from these two animals, none of the virus-treated mice showed any evidence of ascites at the time of death. All the uninfected controls died 11–24 days after transplantation; autopsy of these mice revealed gross distension of the peritoneal cavity by turbid yellow fluid and coating of the viscera with thick creamy deposits of tumor cells and fibrin.

A more detailed description of the results of the first experiment is given in Chart 2 and Table 2. The use of different mice for each tapping probably accounted for some of the variability in the counts. It will be seen (Chart 2) that the percentage of tumor cells began to fall at the end of the first day after virus infection. Simultaneously, the percentage of degenerate cells increased, reaching a peak 10 hours later, and by the end of the 3d day only three tumor cells were observed in a count of 400 cells; of these, one showed advanced degeneration. The duration of the destructive process in this experiment was therefore approximately 24 hours. Differential mitotic counts revealed an increase in the number of grossly abnormal mitotic figures in the virus-treated group (Table 2): the increase, however, was not significant, and the number of gross mitotic abnormalities was very small in both groups. In the few that were present, the aberrations consisted of extensive clumping of the chromosomes in prophase, metaphase, or telophase and, rarely, scattering of the chromosomes in metaphase.

In the virus-treated group, there was a slight increase in the percentages of tumor cells in the later stages of mitosis and a more definite drop in the percentages of cells in prophase (Table 2). The difference between the mean percentages of cells in prophase in the virus-treated and control groups is 2.897 times the standard error of the difference between the two means, and is therefore fairly significant. Application of the rank test (29) to these results indicates that the probability of such a result having arisen by chance is less than 5 per cent but more than 1 per cent. The difference between the mean percentages of cells in the other phases of mitosis in the control and virus-treated groups is not significant.

These results are consistent with the occurrence of a sudden drop in the number of cells entering prophase in the virus-infected tumor, and do not provide any indication of interference with the stages of the mitotic process after the initiation of prophase. The appearance of large numbers of degenerating cells in the virus-infected tumor coincided with a significant fall in the number of mitotic figures (Table 2). Mitoses were observed in only two of eleven smears made on the third to the seventh days after virus inoculation (Chart 2). The degenerative changes would appear, therefore, to affect the tumor cell in interphase and to produce an inhibition of the onset of mitotic division.

Cytological observations of the virus-infected Ehrlich ascites tumor cells.—Microscopical examination of the degenerating tumor cells reveals an exaggeration of the process of multiple nuclear budding occasionally observed in the controls (Fig. 3B). Although all stages of degeneration were seen with the phase contrast microscope, the finest detail was observed under the light microscope in the preparations stained by Barrett's method, upon which most of the description is based.

The earliest abnormality of the virus-infected tumor cell consists of an increase and a rarefaction

of the nuclear sap, which loses its affinity for stain (Fig. 5); simultaneously, the chromatin becomes condensed onto the nuclear membrane and forms a network enclosing the nucleoli (Figs. 4 and 5). The parachromatin granules are replaced by one to six or more pseudonucleoli, the size of which is inversely proportional to their number. One of the largest (1 μ in diameter) is shown in Figure 5.

At the height of the destructive process, pseudonucleoli may be present in as many as 20 per cent of the tumor cells in virus-infected mice. Meanwhile, the true nucleoli become shrunken and angular in shape (Fig. 4) and either divide or disintegrate, so that as many as twenty can be seen in a single nucleus. Throughout this process, the nucleoli retain their associated chromatin and are therefore readily distinguishable from pseudonucleoli.

The periphery of the cell then undergoes multiple budding, each bud containing a nucleolus or a pseudonucleolus. In the present series of experiments, the majority of the nuclei are considerably enlarged before budding occurs; the nuclear membrane is stretched and finally ruptures with the extrusion of multiple club-shaped nucleolar fragments into the cytoplasm (Fig. 6). The end-result resembles a colony of Actinomyces; each club consists of a nucleolus surrounded by its membrane and frequently attached centrally by the remaining chromatin strands of the nucleus. When the nuclear membrane ruptures, the acidophil bodies or naked nucleoli, having no chromatin membrane, are set free into the cytoplasm. Rupture of the nuclear membrane is followed by complete disintegration of the cell.

In a smaller proportion of cells, multiple budding occurs when the nucleus is only slightly enlarged; the nuclear membrane remains intact, the cell does not disintegrate so readily, and the process of amitotic nuclear division is more frequently completed. The resultant number of small "nuclei" is so excessive that fragmentation of the whole cell occurs: when very small, the nucleoli are not visible in cells which have dried before fixation, and the appearance is that of a typical karyorrhexis (Fig. 6). The number of cells apparently showing karyorrhexis in the smears can be increased by allowing them to dry for a few seconds before immersion in the fixative.

During the process of nuclear disorganization, the cytoplasm is increased in proportion to the amount of nuclear enlargement; it stains less strongly (Fig. 5) and contains an increased number of large neutral fat globules. The lipochondria and mitochondria are unaffected and continue to stain supravitally until the terminal stage of nuclear rupture and fragmentation of the cell. The persistence of the supravital staining properties of these structures and the absence of supravital staining in the rest of the cell until the final stage of nuclear disintegration indicate that the cells remain viable up to this point.

The resulting fragments of tumor cells are phagocytosed by large numbers of macrophages which appear in the exudate, and finally the fluid is absorbed.

DISCUSSION

There are many possible ways in which virus infections could influence tumor growth. They could produce vascular lesions which result in ischemic necrosis. By initiating an inflammatory reaction they could bring about an absolute or relative reduction in the number of tumor cells; phagocytosis, enzymatic digestion, alteration of electrolyte balance or osmotic pressure, and the toxic action of the products of tissue destruction may all contribute. The presence of inflammatory cells and exudate could bring about a relative reduction in the number of tumor cells. In the case of tumors, such as the Ehrlich ascites tumor, alteration in the ascitic fluid could result in agglutination or precipitation of the malignant cells. The effects of viruses may resemble those of many physical and chemical agents in producing a nonspecific degeneration involving neoplastic and other cells; or they might possibly induce an increasing differentiation of the tumor. Lastly, and of most interest to us, viruses could multiply selectively in the tumor cells and produce specific changes in them leading to their destruction.

Our experiments show that Bunyamwera virus multiplies in the Ehrlich ascites tumor and that multiplication of virus is accompanied by a selective destruction of tumor cells. The destructive process affects the intermitotic phase of the tumor cell and consists of a form of cellular hypertrophy and abnormal amitotic division leading to total disruption of the cell.

The significance of the development of prominent pseudonucleoli in the virus-infected tumor cells is not clear; their rarity in uninfected tumor cells precludes a more complete study of the fate of cells which contain them. Their resemblance to nucleoli and the fact that their appearance precedes the splitting or division of nucleoli in the virus-infected cells suggests that they are either abortive attempts at nucleolar formation or that they are a side-product of nuclear histone synthesis during the duplication of true nucleoli.

Virus infection produces hypertrophy and abortive hyperplasia similar to, but much more

pronounced than, that which only occasionally occurs in uninfected tumor cells. The oncolytic action of Bunyamwera virus is in no way different from the hypertrophic hyperplastic effects of many other viruses on normal tissues, and the outcome is likewise conditioned by the proliferative capacity of the infected cell (24).

SUMMARY

1. Several features of the morphology and cytochemistry of the Ehrlich ascites tumor cell have been analyzed.

2. The occurrence of nuclear budding or amitotic nuclear division and the development of acidophil intranuclear bodies from the nuclear parachromatin have been described. These bodies are only rarely observed in the uninfected tumor cells, and they have been shown to be chemically and functionally related to nucleoli; it is proposed to call them pseudonucleoli or parachromatin bodies.

3. Bunyamwera virus infection of the Ehrlich ascites tumor results in a selective destruction of the tumor cells. The destructive process involves the intermitotic phase of the cell; it is characterized by the development of prominent pseudonucleoli in a large proportion of infected cells and by the occurrence of multiple nuclear budding similar to, but more pronounced than, that which is occasionally observed in the uninfected tumor cell.

ACKNOWLEDGMENTS

The authors are indebted to Mrs. Marion Greene and Mr. Donald Hendrickson for their valuable technical assistance, and to Mr. Leslie McWilliam for the photography.

ADDENDUM

Since this manuscript was submitted, our attention has been drawn to a paper by Cain (A. J. Cain, The Use of Nile Blue in the Examination of Lipoids. Quart. J. Microscop. Sc., **88**:383–92, 1947), indicating that lipoids staining red with Nile Blue are not necessarily unsaturated. The large sudanophilic droplets in the cytoplasm therefore contain saturated or unsaturated neutral lipid.

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Fig. 1.—Uninfected Ehrlich ascites tumor cells. Leishman stain. ×1,250.

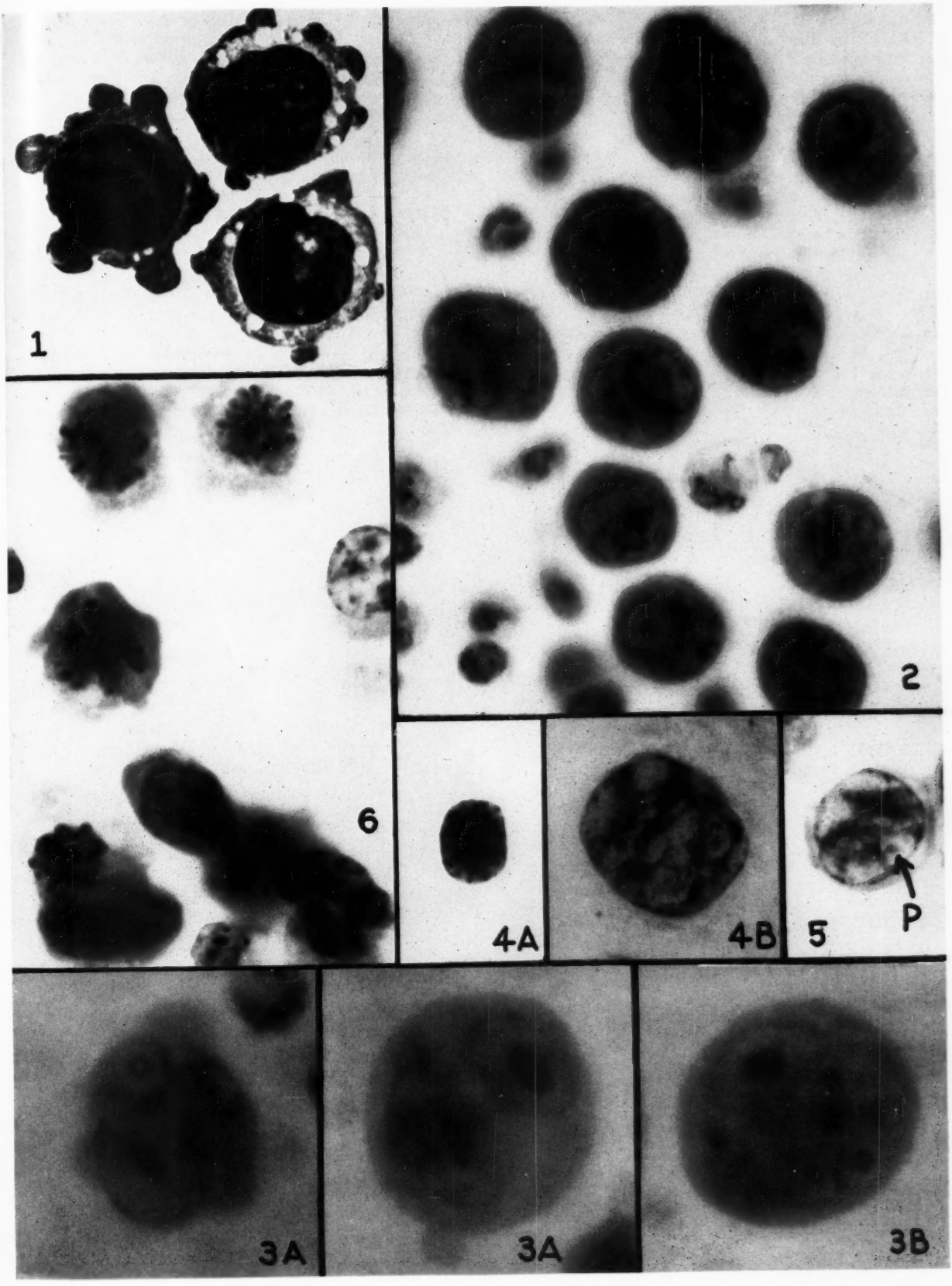
Fig. 2.—Uninfected Ehrlich ascites tumor. Barrett's stain. ×1,250.

Fig. 3.—Uninfected tumor cells in the process of nuclear budding. *A*. Two stages in the formation of two micronuclei from pseudopodia. *B*. Multiple nuclear budding with partial rupture of the nuclear membrane. Barrett's stain. ×1,800.

Fig. 4.—*A*. An uninfected tumor cell. *B*. Early stage of nuclear disorganization. Feulgen stain. ×1,250.

Fig. 5.—Infected tumor cell containing a prominent pseudonucleolus (P). Barrett's stain. ×1,250.

Fig. 6.—Infected tumor cells showing different stages of degeneration. The karyorrhexis in the bottom right hand corner is probably an artifact due to partial drying of the cell before fixation. Barrett's stain. ×1,250.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A Decrease in Plasma Sulfhydryl Content in the Dog after Intravenous Administration of 3,4-Benzpyrene*†

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The implication that sulfhydryl groups are concerned with the carcinogenic process is currently receiving much attention. Polycyclic carcinogenic hydrocarbons have been presumed to interfere with some phase of sulfur metabolism related to growth (2). If a carcinogen such as benzpyrene has the capacity to combine with or cause an oxidation of sulfhydryl groups, a decreased sulfhydryl titer should be detectable in the blood plasma. Not only does the plasma contain freely-reacting sulfhydryl groups, but also the sulfhydryl level in a general way reflects the total thiol content of the tissues.

In the present study, aqueous dispersions of benzpyrene or anthracene were injected intravenously into rabbits and dogs; the changes in the sulfhydryl content of the plasma were followed by an amperometric method (9). Large amounts of benzpyrene produced an average decrease of 17.5 per cent in the sulfhydryl content of the plasma, while comparable amounts of anthracene showed no effect.

MATERIALS AND METHODS

The polycylic hydrocarbons used in this study were 3,4-benzpyrene (BP) (Edcan Labs.), m.p. 179.5° C., corr., and anthracene (AN) (Eastman Kodak), white label, m.p. 217° C., corr. BP was further characterized by its ultraviolet absorption spectrum and filter paper chromatography. No evidence of impurities was found. These hydrocarbons were stored in green glass bottles under ordinary laboratory illumination.

The hydrocarbons were administered intravenously as aqueous colloidal suspensions dispersed in 1 per cent Demal-14 (Emulsol. Corp.), prepared as follows: a weighed amount of BP (3.3 mg/kg body weight) or an equivalent amount of AN (2.3 mg/kg) was dissolved in a minimal amount of acetone (c.p.) with gentle heating. The acetone solution was then drawn into a hypodermic syringe and forcibly ejected, through a No. 20 needle, below the surface of a calculated volume (1.65 ml/kg) of 1 per cent Demal-14. Acetone was removed by boiling the solution under reduced pressure for several minutes. The resulting colloidal dispersion was stable at room temperature for several hours. Demal-14, a polyglycerol ester of oleic acid, has been reported to be nonhemolytic and to have no physiological effects upon laboratory animals in the concentrations employed (3).

The sulfhydryl (SH) content of 1-ml. aliquots of plasma or serum was determined by the amperometric titration technic, as modified by Weissman, Schoenbach, and Armistead (9). Commercial nitrogen, freed of traces of oxygen by suitable absorption traps, was bubbled into the sample undergoing titration; this procedure aided in stirring the solution and also increased the precision of the end point.

Young, adult, male rabbits were utilized in several preliminary experiments. These animals were maintained on Ballard rabbit pellets. The serum SH content of the rabbits was determined every 3 or 4 days over a period of 2 weeks to establish a control SH level prior to intravenous injection of the hydrocarbon.

More extensive studies were carried out on dogs. The dogs employed in this study were maintained on Purina Checkers and water supplied ad libitum. The plasma SH content of each animal was determined at periodic intervals during a conditioning period of 1 month to establish a control SH plasma level.

The general procedure for each experiment was as follows: the dog was anesthetized with sodium pentobarbital (30 mg/kg); heparinized control blood samples were obtained 1 and 2 hours after induction of anesthesia for determination of hematocrit, plasma SH, and serum nitrogen partition.

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The animal was then given a single intravenous injection of BP or AN dispersed in 1 per cent Demal-14; blood samples were obtained within the 1st hour and at convenient intervals after the administration of the hydrocarbon for hematocrit, plasma SH, and serum total nitrogen determinations. In one experiment (F) the calculated amount of BP was given in three divided doses, rather than as a single injection.

Although each animal served as its own control, an additional control experiment (A) was performed in which the effect of doubling the volume of 1 per cent Demal-14 was observed. During the conditioning periods, it was established that the anesthetic had no effect on the plasma SH content.

Total N, NPN (nonprotein nitrogen, Folin-Wu filtrate), and albumin N (serum fractionated by the methanol precipitation method of Pillemer and Hutchinson [6]) were determined by micro-Kjeldahl method. The amount of fluorescent material in the dog plasma before and after intravenous injection of the hydrocarbon was determined with a Coleman photofluorometer.

RESULTS

In the preliminary experiments, one rabbit showed a 27 per cent decrease in serum SH content 1 hour after a single intravenous injection of 5 mg. of BP dispersed in 5 ml. of 1 per cent Demal (control serum SH content was 31 μM per cent; 23 μM per cent after BP); another rabbit showed a 16 per cent decrease in serum SH content after receiving 10 mg. of BP dispersed in 10 ml. of 1 per cent Demal.

The effects of single and divided intravenous injections of BP, AN, and Demal-14 on the plasma SH content of the dog are summarized in Chart 1. Single injections of BP produced a significant decrease in dog plasma SH content within ½ hour, and this effect persisted for at least 6 hours. An equivalent amount of BP administered as three divided doses (Exp. F) did not produce as pronounced an effect as did a single injection. Injections of AN did not produce a significant decrease in the plasma SH content of dogs. Control values from anesthetized and nonanesthetized dogs were the same. The dispersing agent, 1 per cent Demal-14, had no effect upon plasma SH content of an anesthetized dog (Exp. A).

Serum N partition (Table 1) indicated that intravenous injection of either BP or AN generally was accompanied by a decreased total serum N and an increased A/G ratio. When the SH content of dog plasma is related to total serum N (Table 2), anthracene injection is accompanied by an increase in μM of SH/mg N, while in every case BP injection is associated with a decrease from control values.

No significant change in the amount of fluorescent material usually present in normal dog plasma was obtained within ½ hour after intravenous administration of either hydrocarbon.

DISCUSSION

The decreases in plasma sulfhydryl content observed in these experiments obviously cannot of themselves account for the carcinogenic effect of

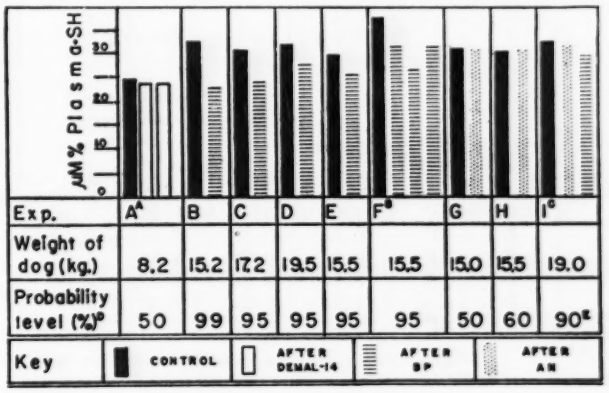

CHART 1.—The effect of intravenous injections of anthracene, 3,4-benzpyrene, or 1 per cent Demal-14 on dog plasma SH content.

A.—Two single injections, given 2 hours apart, of 1 per cent Demal-14 only (twice the calculated volume used throughout the remaining procedures).

B.—The calculated amount of benzpyrene was given in three divided doses instead of a single injection.

C.—The calculated amount of benzpyrene was given about 9 hours after injection of the same dog with anthracene.

D.—Determined by the *t* test.

E.—Value after benzpyrene injection.

The sulfhydryl content of dog plasma was obtained over a 2–3-hour period for the control level and over a 4–6-hour period after intravenous injection of the hydrocarbon.

benzpyrene. These results do stand as direct confirmation of the theory that carcinogenic hydrocarbons can affect sylfhydryl groups. Under comparable conditions, a noncarcinogenic hydrocarbon, anthracene, was ineffective in reducing SH titer of dog plasma. From a biochemical point of view, the benzpyrene effect may reflect a general pattern of reaction by which specific cellular enzymes controlling growth are altered in their activity. Of possible significance to this line of reasoning are a number of observations that cancer patients have low sulfhydryl plasma values (1, 7, 8).

The amounts of hydrocarbon injected were 8–10 times the molar equivalent of the estimated sulfhydryl groups of the plasmas of the dogs. However, several factors limited the molar reactivity of the benzpyrene *in vivo*. The compound

was dispersed as a colloid, it was rapidly removed from the blood stream and presumably was metabolized (5), and the body undoubtedly utilized its capacity to regenerate obliterated sulfhydryl groups.

The observations presented offer no explanation of the mechanism of the effect. The slight changes in plasma proteins have not been correlated with decreases in sulfhydryl titer. *In vitro* incubation of dog or rabbit sera with benzpyrene produced no decrease in the sulfhydryl content. An inhibition of urease by light-activated benzpyrene has been reported (4). The inhibition was reversed by cysteine, and the benzpyrene lost its inhibitory power during storage in the dark. The benzpyrene injected intravenously in the present study was pure by ordinary chemical tests and was handled in the usual manner without protection from ordinary laboratory illumination. Thus, the effect on the sulfhydryl content of plasma can best be regarded as being produced by the kind of benzpyrene preparation which numerous biological tests have shown to be carcinogenic.

SUMMARY

Intravenous injections of aqueous colloidal dispersions of 3,4-benzpyrene produced in rabbits and dogs a significant decrease of serum or plasma sulfhydryl content which persisted for a considerable period of time after removal of the benzpyrene colloid from active circulation. This effect was not produced by molecularly equivalent amounts of anthracene administered in the same manner.

TABLE 1

THE EFFECT OF BENZPYRENE OR ANTHRACENE ON SERUM NITROGEN OF THE DOG

Exp.	Compound injected	Control (1 hr. after anesthesia) mg N/ml Total	NPN	Alb.	Glob.	A/G Ratio	Experimental (4 hr. after hydrocarbon administration) mg N/ml Total	NPN	Alb.	Glob.	A/G Ratio
C	BP	12.01	0.26	4.48	7.32	0.61	11.87	0.26	4.70	6.91	0.68
D	BP	10.56	0.28	4.09	6.18	0.66	10.15	0.34	4.56	5.25	0.87
E	BP	9.03	0.29	4.69	4.05	1.16	8.95	0.27	4.45	4.23	1.05
H	AN	9.10	0.30	2.95	5.84	0.51	8.53	0.34	3.03	5.16	0.59
I	AN	11.21	0.25	5.08	5.88	0.86	9.97	0.23	5.52	4.22	1.31
	BP*						9.97	0.28	5.21	4.48	1.16

* Effect of benzpyrene upon serum N 1 hour after injection.

TABLE 2

THE RELATION BETWEEN PLASMA SH AND SERUM N CONTENT

Exp.	Compound injected	Control period μM SH/ml*	μM SH/mg N	After hydrocarbon administration μM SH/ml*	μM SH/mg N
C	BP	0.312	0.026	0.245	0.024
D	BP	0.327	0.031	0.280	0.028
E	BP	0.300	0.033	0.260	0.029
H	AN	0.310	0.034	0.317	0.037
I	AN	0.332	0.030	0.325	0.033
	BP†			0.305	0.030

* μM SH/ml plasma—average values.
The SH values used in the calculation of μM SH/mg N are the average values obtained during the control period (two samples; four determinations) and the experimental period (two samples; four determinations).

† Sample obtained about 9 hours after intravenous injection of the same dog with anthracene but within 1 hour after injection of benzpyrene.

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Assay of Spontaneous and Transplanted Mammary Tumors for the Mammary Tumor Agent*

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The mammary tumor agent, one of the primary causes of mammary cancer in mice (5, 6), has the characteristics of an infectious agent or virus.

The presence of the agent in infected hosts may be demonstrated by making extracts of either normal or cancerous tissues and injecting these into susceptible animals which do not carry the agent. It has been assumed that the percentage of mice developing tumors and the average cancer age are indicative of the concentration and/or activity of the agent.

In several studies (2, 4, 9, 11) transplanted tumors have been tested for the presence and propagation of the mammary tumor agent. However, in most studies the activity of the agent from transplants of one tumor was compared to that from spontaneous tumors which developed in other animals of the same or a different cancerous stock. Such results do not give definite information as to the best source of the agent for biological work, especially where large amounts of material may be required.

When the concentration and/or activity of the agent from transplanted and from spontaneous mammary tumors are compared, the data should be based upon studies where spontaneous and transplanted tumors descended from the same tumor are used. By doing so, information may be obtained on the survival of the agent in the transplants over a number of passages.

An attempt has been made to do so in this report, where the final observations (10, 11, 13) are presented on the tumor-inducing ability of the agent secured from several spontaneous mammary tumors and from the transplants of those which were continued for a number of passages in mice which did not possess the agent. In some experiments, normal tissues were also assayed from the same donors which were bearing either spontaneous or transplanted mammary tumors.

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MATERIALS AND METHODS

Five spontaneous adenocarcinomas of the mammary gland, which developed in breeding females, were used, of which three were transplanted by the trocar technic.

Tumor No. Z8044 appeared in a female of the cancerous Z (C3H) stock when the animal was 278 days of age. The mouse was a member of the 53d successive generation to have mammary cancer.

The other four tumors developed in females of the B (C57BL) stock, subline 6, either in mice which had been fostered by females of cancerous strains or in their progeny.

Tumors Nos. B7478z_1 and B7788z_1 were from B females which had been nursed by females of the Z stock, and the tumors were observed when the mice were 482 and 500 days of age, respectively. Litter-mates give rise to the other two tumors, Nos. B7476a_2 and B7477a_2, at the respective ages of 343 and 320 days. Their mother, fostered by a female of the A stock (A_1), also had a mammary cancer when she was 287 days of age.

The tissue to be assayed was weighed, ground in a mortar with sand, and suspended in either physiological saline or distilled water, 1:10. Distilled water was used except for the four spontaneous tumors from the B females. The tumor suspensions were centrifuged twice, for periods of 10 minutes each, at approximately 2,500 r.p.m. The final supernatant was removed and either used at the same concentration or diluted so that the intraperitoneal injection of 1 cc. contained the agent extracted from an equivalent weight of tumor tissue as given in the tables.

The test animals were ZBC hybrids, derived by matings between mice of the fostered (without the agent) lines of the A and Z stocks (11), with an incidence in controls of less than 1 per cent. The age of the mice at the time of the administration of extracts of tumor Z8044 are given in the table; in the other studies they were from 20 to 27 days. All experimental mice were continued as breeders, and few of those which died noncancerous had less than five litters. Only noncancerous mice which survived for longer than 250 days have been included in the tabulations.

The mice were maintained on Purina Fox Chow and tap water.

RESULTS

The results obtained following the injection of extracts of the five spontaneous mammary tumors are recorded in Table 1.

TABLE 1

BIOLOGICAL ASSAY IN ZBC HYBRID MICE FOR THE MAMMARY TUMOR AGENT IN SPONTANEOUS MAMMARY TUMORS FROM THE B (C57BL) AND Z (C3H) STOCKS

Tumor no.	Gm. equiv. injected	No. mice	Per cent cancer	Av. age in days With cancer	Av. age in days Without cancer
B7476a*	2×10^{-2}	20	80	455	609
	10^{-3}	13	46	429	531
B7477a_2	2×10^{-2}	29	59	373	456
B7477a_2	10^{-3}	25	84	498	628
B7478z_1	10^{-2}	31	81	461	486
B7788z_1	2×10^{-2}	41	95	350	690
	10^{-3}	49	88	352	474
Z8044	10^{-2}	38	76	328	468
	10^{-4}	33	70	311	528

* A (a) or Z (z) agent, and generations in B stock.

and had been transplanted for 41 passages when the extensive amount of necrosis in the tumors discouraged further study. The hosts were F_1 hybrids, produced by reciprocal matings between fostered A and Z animals, and the grafts grew progressively in all.

The intraperitoneal injection of extracts of tumor Z8044, of either the spontaneous or transplanted tumors, gave the results recorded in Table 3, where the data are arranged according to the concentration of the extracts. As noted, two series were tested with transplants of the first passage using tumors either 31 or 53 days following inoculation. Details about the data will be considered later.

The liver and spleen from the cancerous mouse bearing tumor Z8044 were also extracted and tested for the activity of the mammary tumor agent. Owing to the number of fractions that were used in this study, different groups of mice were injected with the tumor extracts to ascertain if there might be any loss of activity during the time required for administration. These are listed in the order they were injected in the tabulations (Table 4). The first and seventh series received the amount of the agent derived from 10^{-4}-gm. equivalents of tumor tissue, and no significant variation was observed in the results. This concentration of tumor was as active as the extract containing 10^{-2}-gm. equivalents of tissue. At a dilution of 100-fold, the tumor-inducing ability of spleen was approximately the same as that of the tumor; when 10^{-4}-gm. equivalents were used, the activity of the former was greatly reduced. At all dilutions tested, extracts of liver were much less active than the other tissues obtained from the cancerous donor.

Thirty-one days following the first passage of tumor Z8044, at the time the transplanted tumors were assayed, spleen and liver from tumor-bearing mice were also used. As compared to the transplanted tumors, spleen and liver were relatively inactive (Table 5).

TABLE 2

ASSAY IN ZBC HYBRID MICE FOR THE AGENT IN SPONTANEOUS AND TRANSPLANTED MAMMARY TUMORS FROM B FEMALES

Tissue extracted	Gm. equiv. injected	Tumor B7476a No. mice	Tumor B7476a Per cent cancer	Tumor B7476a Av. age in days With cancer	Tumor B7476a Av. age in days Without cancer	Tumor B7477a_2 No. mice	Tumor B7477a_2 Per cent cancer	Tumor B7477a_2 Av. age in days With cancer	Tumor B7477a_2 Av. age in days Without cancer
Spontaneous	2×10^{-2}	20	80	455	609	29	59	373	456
	10^{-3}	13	46	429	531	25	84	498	628
Transplants, first passage	2×10^{-2}	9	78	420	537	29	100	371	
	10^{-3}	21	52	450	506	20	80	457	558
Transplants, tenth passage	10^{-1}	38	92	400	512	38	89	339	521
	10^{-3}	44	73	475	600	44	55	451	624

The earliest average cancer ages to be found in the injected ZBC mice were seen in those which received extracts of the mammary tumor from a female of the cancerous Z stock, Z8044; the next for one tumor from a B mouse with the agent from the Z stock (B7788z_1). Considerable variation was encountered when the two tumors from females of the B stock with the agent derived from the A strain were assayed, although these tumors developed in litter-mates and were tested the same day. However, at some concentrations, extracts of these tumors were as active as another cancer with the Z agent, B7478z_1.

Transplants of the first and tenth passages of tumors B7476a_2 and B7477a_2 were also studied, and the data have been tabulated in Table 2. That the tumors possessed the mammary tumor agent, after being transplanted for ten passages in mice of the B stock, is evident from the results.

Tumor Z8044 was continued for 23 months through 30 transplant generations to investigate the propagation of the mammary tumor agent,

DISCUSSION

Since 1944 (8), it has been known that the mammary tumor agent could be recovered from transplanted mammary tumors, even after ten (10–13, 17), 30 (13), and 42 (15) serial passages in animals which did not themselves carry the agent. Although the results obtained by various workers have not been in accord as to the advisability of employing this material as the source of the agent for various problems, only preliminary data (10, 11, 13) have been presented on some serial dilution studies comparing the activity of the agent in spontaneous mammary tumors, and in transplanted tumors descended from the same.

Andervont (1) reported that, although a spontaneous mammary tumor from a C3H female and the fourth passage transplants carried in C3H hosts possessed the agent, when this tumor was transplanted into agent-free hybrids, the third and 23d transfer tumors failed to induce mammary tumors in suitable test animals, from which it was concluded that the agent was absent in these transplants. Dmochowski (14) was unable to demonstrate the agent in the transplants of the 428th passage of the Imperial Cancer Research Fund tumor No. 63.

Four spontaneous mammary tumors from B (C57BL) females were assayed for the agent in the present study. Two tumors arose in females which had been nursed by females of the Z (C3H) cancerous stock, and the others arose in litter-mates born to a mother which had been fostered by a female of the cancerous A stock. That the spontaneous tumors did not possess the same degree of activity, due to the presence of the agent, is evident from the results (Table 1). The one with the greatest tumor-inducing ability in the test animals, B7788z_1, appeared in a B mouse with the Z agent, and yet another tumor (B7478z_1) from a mouse fostered by a female of the same strain was no more active than a tumor with the A agent after it had been propagated for one generation in mice of the B stock.

The time of appearance of the spontaneous mammary tumors in the B mice did not bear any relationship to the activity of its mammary tumor agent, since the tumors with the A agent developed

TABLE 3

ASSAY OF TUMOR Z8044, WITH EXTRACTS FROM THE SPONTANEOUS AND TRANSPLANTED TUMORS

The data are arranged according to the concentration of the extracts administered.

Passage	Gm. Equiv. injected	Age when injected	No. mice	Per cent cancer	Av. age in days With cancer	Av. age in days Without cancer
24th	10^{-1}	23–25	23	87	325	528
7th	2×10^{-2}	25–27	23	78	347	438
Sp. T.*	10^{-2}	24–26	38	76	328	468
1st†	10^{-2}	22–26	42	67	343	438
1st	10^{-2}	17–19	15	93	304	350
1st	10^{-2}	23–26	21	86	299	408
18th	10^{-2}	27–29	25	36	435	520
20th	10^{-2}	20–23	35	80	359	621
24th	10^{-2}	23–25	26	96	333	380
30th	10^{-2}	20–27	50	80	379	519
7th	10^{-3}	25–27	49	55	363	487
24th	10^{-3}	23–25	31	58	386	624
Sp. T.	10^{-4}	22–26	33	70	312	528
1st†	10^{-4}	20–21	26	88	348	567
1st	10^{-4}	17–19	18	72	360	399
1st	10^{-4}	23–26	20	65	357	451
18th	10^{-4}	27–29	22	9	287	599
20th	10^{-4}	20–23	37	24	416	564
24th	10^{-4}	23–25	30	33	387	599
30th	10^{-4}	20–27	40	18	434	655
1st	10^{-5}	17–19	19	32	398	534
1st	10^{-5}	23–26	28	46	345	446
18th	10^{-5}	27–29	22	4	318	557
20th	10^{-5}	20–23	32	22	447	680
24th	10^{-5}	23–25	26	4	287	637
30th	10^{-5}	23–25	60	5	608	664
20th	10^{-6}	20–23	34	6	397	626
30th	10^{-6}	20–27	47	0		621

* Sp. T. = spontaneous tumor.

† Used 31 days after inoculation; others same passage after 53 days.

TABLE 4

ASSAY FOR THE AGENT IN THE SPLEEN AND LIVER FROM THE MOUSE BEARING SPONTANEOUS MAMMARY TUMOR Z8044

The groups are listed in the order they were injected; test ZBC mice were 22–26 days of age.

Order of inj.	Tissue assayed	Gm. equiv. injected	No. mice	Per cent cancer	Av. age in days With cancer	Av. age in days Without cancer
6th	Tumor	10^{-2}	21	76	318	421
8th	Tumor	10^{-2}	17	76	339	527
Total			38	76	328	468
1st	Tumor	10^{-4}	18	67	321	518
7th	Tumor	10^{-4}	15	73	302	544
Total			33	70	312	528
5th	Liver	10^{-2}	17	24	280	420
2d	Liver	10^{-4}	22	9	509	550
4th	Spleen	10^{-2}	19	68	306	468
3d	Spleen	10^{-4}	22	32	331	530

TABLE 5

TESTS OF SPLEEN, LIVER, AND TRANSPLANTED Z8044 TUMORS FROM MICE 31 DAYS AFTER INOCULATION OF THE FIRST TRANSPLANT GENERATION

ZBC Test mice

Tissue assayed	Age when injected	Gm. equiv. injected	No. mice	Per cent cancer	Av. age in days With cancer	Av. age in days Without cancer
Spleen	22–25	10^{-2}	20	15	513	602
Spleen	22–25	10^{-4}	27	4	433	557
Liver	22–25	10^{-2}	15	13	521	620
Liver	20–21	10^{-4}	29	7	346	627
Tumor	24–26	10^{-2}	42	67	343	438
Tumor	20–21	10^{-4}	26	88	348	567

before the mice were 350 days of age, while the most active tumor, B7788z_1, appeared when the mouse was 500 days of age. In some tests, the amount of the agent derived from 10^{-3}-gm. equivalents of tissue produced a higher frequency in the test mice than did extracts containing a larger amount of material. While the extracts of the tumor from the Z stock, Z8044, did not cause more of the ZBC test mice to become cancerous, their tumors appeared at an earlier average age than was seen following the administration of extracts of the tumors from the B mice.

The two mammary tumors from litter-mates of the B stock possessing the A agent were transplanted for ten passages in unfostered B mice. Since the spontaneous tumors and transplants of the first and tenth passages were tested for the agent, these data, at some dilutions, may be compared for activity (Table 2). At all concentrations, extracts of the first-passage tumors were as active as the spontaneous tumors; after ten passages, some were more active, others perhaps less active.

With the exception of the eighteenth passage, extracts of the transplants of the Z tumor, Z8044, that were tested between the first and 30th passages showed approximately the same tumor-inducing ability at a dilution of 100-fold as did the spontaneous tumor, and comparable results were obtained for transplants of the seventh and 24th passages with 10^{-3}-gm. equivalents of tissue. When dilution was 10,000 times, there was little difference between the activity of extracts of either the spontaneous tumor or transplants of the first passage, and an extract of the latter containing 10^{-5}-gm. equivalents produced an incidence of 43 per cent in the test animals (Table 3).

All extracts of transplants of the eighteenth passage, regardless of the amount of material administered, showed lower activity, and it was not restored in later transplants except when the concentration was no more than 10^{-3} gm. equivalents. Some data for tumors of the seventh passage suggested that this "decrease in activity" might have occurred earlier.

Some variation was encountered when transplants of the same passage, the first (Table 3), were tested at different intervals following inoculation, and these results could not be correlated either with the concentration of the extract, the size of the tumors, or a slight difference in the age of the mice at the time of treatment.

Extracts of spleens from cancerous donors were found to be a poorer source of the mammary tumor agent, in general, than most spontaneous and transplanted tumors have been. When the spontaneous tumor, Z8044, and the spleen and liver from the cancerous animal were tested, spleen and tumor showed approximately the same tumor-inducing ability when the extracts were diluted 100-fold. If the extracts contained 10^{-4} gm. equivalents, the activity of the tumor remained approximately the same, while that of spleen was greatly reduced at this concentration. At both dilutions, liver proved to be relatively inactive (Table 4).

Previously, it was found (7) that, after small pieces of spleen from young donors were grafted, the presence of the agent in the grafts produced mammary cancer in over 20 per cent of the inoculated BAF_1 females, and the agent was transferred by the inoculated females to their progeny. The agent could also be transferred by the transplantation of other normal tissues.

Prehm (18) has recently reported upon the transfer of the agent by the inoculation of either normal or cancerous tissue. When spleens from mice which possessed the agent were grafted, in several series at least 40 per cent of the females later developed mammary tumors, and others became "infected." The agent could also be recovered from the spleens of the inoculated animals and could be detected within a period of 3 weeks, or earlier, after the grafts had been transplanted.

The spleens and livers from mice bearing the first-passage transplants of tumor Z8044 were tested 31 days following the inoculation of the tumors. Extracts of these tissues showed little activity, and the low incidences, together with the late average cancer ages, suggested that the agent probably could not be demonstrated (Table 5). At a concentration of 10^{-4}-gm. equivalents, the tumor produced an incidence of 88 per cent in the test mice.

By the subcutaneous transplantation of mammary tumors to susceptible animals, and by excising tumors after 26–53 days, Andervont (2) tested this technic for the transfer of the agent from the tumor to the inoculated host. Only 4 per cent of the animals later had mammary tumors. Hummel and Little (17) used a similar method but used the ear for the inoculation site, and, after the tumors had developed, the ears were amputated. Fifty-two per cent of the inoculated hybrids, BAF_1 (B♀ × A♂), which received transplants of the first, fifth, and tenth passages of a tumor from the A stock showed progressively growing tumors. Among this group, 6 per cent had mammary cancer, while of those in which the ear transplants regressed, the incidence of mammary cancer was found to be 13 per cent. Extracts of the transplants, of unstated concentration, were also administered, and evidence was obtained that the agent had survived ten passages.

A higher incidence was observed by Prehm (15) following the removal of subcutaneous transplanted tumors, for in one series 40 per cent later gave rise to spontaneous mammary cancer. In a group of seven hybrids resistant to grafts of the tumor, six had mammary cancer due to the transfer of the agent from the transplanted graft.

In a previous publication (3), an attempt was made to ascertain the most active source of the mammary tumor agent by comparing the results found in test animals following the injection of extracts of various tissues. With material from donors of the A stock, lactating mammary glands were found to have a greater tumor-inducing potency than did spontaneous mammary cancer, and spontaneous mammary cancer was a better source of the agent than were transplants of the first passage. Lactating mammary glands from females of the Z (C3H) stock were less potent than mammary glands from the A strain. ABC mice were used as test animals for these studies, derived by matings between mice of the B and fostered A (Ax) stocks.

The evaluation of the observations on the concentration and/or activity of the mammary tumor agent, obtained following biological assay, must be made with many factors in mind.

The tumor-inducing ability either of the same material from comparable donors of the same inbred stock, as cancerous donors (Table 1), or of different tissues from the same donor (Tables 4 and 5), need not be the same, even when assayed in litter-mate controls. Also, when extracts of the same tissue have been tested by serial dilution, the amount of the agent to be administered may not be the determining factor, as has been shown in several studies (see 3, 4, 11).

Likewise, it has been demonstrated in reciprocal crosses between different inbred cancerous strains that the mammary tumor agent transferred by females of these stocks need not have the same activity in offspring with the same genetic make-up. When females of the same maternal strains are used to produce hybrids with other genetic constitutions, different incidences and average cancer ages may be obtained (see 11). This would imply that, to assay the tumor-inducing activity of material from two or more strains, test animals should be used which bear the same genetic relationship to the donor strains; and, based upon the results of using several tumors from a single stock (Table 1), multiple tests should be made.

It seems probable that adequate tests have not been completed, to date, to assay various tissues from either the same or different inbred stocks as to the best source of the mammary tumor agent for experimental studies. Because of the individual variation between the material tested in these studies, either tissues from the same donor or comparable tissues from litter-mates, it is doubtful if comparisons of all these data would be meaningful; for this reason, each study should be considered separately.

It is to be recognized that aqueous extracts of tissues may carry inhibiting and/or inactivating factors for the mammary tumor agent. For example, intracellular enzymes liberated by maceration may influence the amount of activity shown by an extract.

Under the experimental conditions outlined in this report, it seems probable that for the first passage and, perhaps, for ten passages, transplanted mammary cancer may possess the mammary tumor agent with activity equal to the original spontaneous mammary cancer. For some studies, where a large amount of material with approximately the same tumor-producing activity may be needed over a period of days, transplanted mammary cancer might be the most satisfactory source of the agent for biological studies.

SUMMARY

In biological assays on the tumor-producing ability of the mammary tumor agent, considerable variation was observed in the activity of several spontaneous mammary tumors from the same inbred stock, including those from litter-mates.

The results could not be correlated with either the source of the mammary tumor agent or the time of development of the tumors.

Extracts of three transplanted tumors were as active after one passage, and in some cases ten, as the original spontanecus tumor.

A decrease in the activity of one transplanted tumor was seen in the eighteenth passage, and it was not restored in later passages except when extracts of high concentrations were administered.

The mammary tumor agent survived for as long as the tumors were transplanted—namely, ten and 30 passages.

Spontaneous mammary cancer proved to be a better source of the mammary tumor agent than did either spleen or liver from the cancerous donor.

In one study, it is probable that the mammary tumor agent could not be demonstrated in the liver and spleen from donors bearing transplanted tumors, although the tumors were very active.

For some experiments, transplanted mammary cancer may be the most satisfactory source of the mammary tumor agent.

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Metabolism of Neoplastic Tissue. V. Fatty Acid Oxidation in Slices of Transplanted Tumors*

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A number of reports have appeared in recent years which indicate that fatty acids may not be metabolized by neoplastic cells (1, 3, 5, 10), but, in general, little attention has been given to this problem. When recent studies with isotopic tracers (8, 15, 16) demonstrated that tumor slices *in vitro* can oxidize a variety of carbohydrate intermediates and fatty acids, a study of fatty acid oxidation in neoplastic tissue was undertaken. In the present study, data are reported on the oxidation of carboxyl-labeled acetate, butyrate, octanoate, and palmitate in a variety of transplanted tumor slices. Particular attention was devoted to a comparison of liver and hepatoma with respect to ketone body formation and oxidation, since liver differs characteristically from nonhepatic tissue in these metabolic properties.

METHODS

Labeled compounds.—The carboxyl-labeled acids were prepared as described elsewhere (11, 13, 14) from C^{14}-labeled barium carbonate obtained on allocation by the Isotopes Division of the United States Atomic Energy Commission. The labeled acetoacetates were prepared from the respective carboxyl- or methyl-labeled acetates (obtained from Tracerlab, Inc.) according to the procedure already described (11). To insure the complete absence of acetate from the α, γ-labeled acetoacetate, it was purified by the method of Curran (4). Radioactivity measurements were made with a mica-window or a gas flow counter after conversion to barium carbonate by wet combustion (17) with the use of "infinitely thick" samples. The radioactivities of the acids employed ranged from about 800 to 20,000 c.p.m/7.5 sq cm dish. Values for respiratory CO_2 and acetoacetate are given in relative specific activity (R.S.A.) calculated from the equation, R.S.A. = specific activity (s.a.) of product $\times$ 100/s.a. of acid. Total conversion of acids to respiratory CO_2 or to acetoacetate are given in conversion capacity (C.C.). This value represents the microatoms of fatty acid carbon converted to the product in question per hour per gram of tissue dry weight.[1] It is calculated from the equation,

$$\text{C.C.} = \frac{\text{R.S.A.}}{100} \times \frac{\text{microatoms carbon in product}}{\text{hrs.} \times \text{gm. dry wt. tissue}}.$$

Methods of tissue preparation, incubation, isolation of respiratory CO_2 and acetoacetate, and calculations have been described in a previous publication (13).

Tumors.—The solid, neoplastic tissues employed in this study were maintained by subcutaneous transplantation in mice of the A and C3H strains, and in rats of the Fischer line (15). They were used after attaining an approximate diameter of 1 cm., at which time the tissues were firm and resilient, with no macroscopically visible necrosis. The ascites tumors were implanted intraperitoneally, and cells were drawn off after 7–10 days.

RESULTS AND DISCUSSION

A preliminary survey of the ability of various tumor slices to oxidize C^{14}-labeled fatty acids, the results of which are shown in Table 1, revealed

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[1] The calculation of this value is based on the assumption that the R.S.A. represents the relative proportion of substrate carbon in the respiratory CO_2. This is true only if all carbons of the substrates are metabolized in the same manner as the labeled carbons. Previous studies (2) indicate this assumption is reasonably valid. The C.C. has no absolute significance. It has comparative value under comparable conditions, but would be expected to fluctuate widely, particularly with changes in substrate concentration.

that a variety of fatty acids, ranging in chain length from 2 to 16 carbon atoms, undergoes oxidation to CO_2 in a number of transplanted tumors. Fatty acid oxidation appeared to occur at a low rate in tumors, particularly acetate (cf. 8), propionate, and octanoate, but no generalizations were allowable, since the mouse hepatoma oxidized octanoate as readily as did normal mouse liver. A striking result was the high rate of acetoacetate oxidation in tumor slices.

Following this preliminary study, a more detailed investigation was made of the oxidation of the fatty acids, acetate, butyrate, octanoate, and palmitate in transplantable mouse neoplasms. In each experiment a comparison was made between the tumor slices and the non-neoplastic liver of the tumor-bearing animal. The results of this study are collected in Tables 2 to 5. With none of the substrates were any differences apparent between liver slices from normal animals and tumor-bearing hosts. In general, fatty acid oxidation was considerably slower in the tumor slices than in the liver slices, the differences being more marked with the short chain acids than with palmitate. Of the tumors tested the hepatoma invariably had the highest rate of fatty acid oxidation. Lowered fatty acid oxidation in tumor slices is a resultant of two factors: (*a*) a lowered rate of respiration, as indicated by smaller values of oxygen consumption and CO_2 production (columns 2 and 3); and (*b*) a smaller proportion of the labeled substrate carbon in the respiratory CO_2, as indicated by the low values of the R.S.A. in column 4. Thus, not only do the tumor slices display a lower respiratory activity in presence of the fatty acid, but they also have a greater preference for utilization of endogenous carbon.

Though these results could be interpreted as indicating that impairment of fatty acid oxidation may be a feature of the intact neoplasm, there are several reasons why this conclusion may not be warranted. As shown in Table 5, differences in fatty acid oxidation between liver and tumor slices are least with the natural 16-carbon palmitic acid, which most closely resembles the fatty acids available to tumor cells under physiological conditions. A likely cause of the lowered oxidation of short-chain acids is evident on examination of data on octanoate oxidation in Table 4. Not only was octanoate not readily oxidized by tumor slices; the low values for oxygen uptake and CO_2 production, as compared to these values in the presence of other fatty acids, pointed definitely to a pro-

TABLE 1

OXIDATION OF FATTY ACIDS LABELED IN CARBOXYL CARBON BY LIVER AND NEOPLASTIC TISSUES

Approximately 1 gm. (dry weight) of tissue slices were incubated 2 hr. in oxygen at 38° C. with 40 ml. of Ringer-phosphate solution. Substrate concentrations were 0.01 M and values are given as conversion capacity (C.C.).

	Butyrate	Octanoate	Palmitate	Acetoacetate	Acetate	Propionate
Normal rat liver	67	48	10	23	58	50
Host* " "	91	48	9	28	44†	42
Hepatoma, rat	22	8	14	29	4†	5
Ascites hepatoma, rat	15	3				
Host mouse liver		126				
" " kidney		289				
Hepatoma, 7A77, mouse		134				
Mammary carcinoma, mouse		4		83		
Ehrlich ascites tumor, mouse	6, 5					
Rhabdomyosarcoma, mouse	30	4		37		

* Host tissue refers to tissue taken from tumor-bearing animal.

† Animal fasted 24 hours.

TABLE 2

OXIDATION OF ACETATE-1-C^{14} BY LIVER AND NEOPLASTIC TISSUES

Approximately 0.8 gm. dry wt. of tissue slices were incubated with oxygen in 40 ml. of Ringer-phosphate medium at 37° C. Incubation period was 2 hours, with the substrate concentration 0.01 M. All values are calculated on the basis of 1 gm dry wt of tissue/hr of incubation.

	O_2 uptake	Respiratory CO_2		
			R.S.A.	C.C.
	μM	μM	Per cent	μ atoms C
Normal liver	520	337	17.1	57.8
Host liver*	486	350	14.0	49.0
Hepatoma 98/15	341	325	5.8	18.9
Host liver	617	463	17.7	82.0
Mammary adenocarcinoma	151	180	3.3	5.9
Host liver	473	317	12.5	39.7
Sarcoma 37	266	229	2.5	5.6
Host liver	615	448	15.0	67.4
Rhabdomyosarcoma	253	254	2.8	7.2

* Host liver refers to the liver taken from the tumor-bearing animal.

nounced inhibition of the endogenous respiration. Since octanoate is known to be inhibitory at high concentrations in liver homogenates (7), its oxidation by tumor slices was tested at lower concentrations. The effect of a lowered octanoate concentration on its oxidation by tumor slices is strikingly shown in Table 6 where, it will be noted, a tenfold decrease raised its oxidation to levels similar in magnitude to that displayed by normal liver or hepatoma at high octanoate concentration. This matter was not further investigated; however, it seems likely that the low rates of oxidation of short-chain fatty acids can, at least in part, be attributed to inhibitory effects of these substances on respiration. The especial sensitivity of tumor slices may be a reflection of an increased cellular permeability to these substances, resulting in a higher intracellular concentration of the fatty acids.

Conversion of fatty acids to acetoacetate.—In the preliminary survey of fatty acid oxidation, represented in Table 1, attempts were made, without success, to demonstrate acetoacetate formation in tumor slices. Since butyrate is readily converted to acetoacetate in liver (13), it was anticipated that if such conversion occurred in neoplastic tissue slices, it would be most readily demonstrable with butyrate as substrate. In experiments shown in Table 7, carrier acetoacetate was added after incubation and was recovered in the form of its thermal degradation products, acetone and CO_2. As expected from previous work (13), there was a rapid ketogenesis in liver slices, which ranged in magnitude from as high as the CO_2 production to considerably higher. In contrast, ketogenesis in the tumor slices, including the hepatoma, was very low. In all instances the activities were so low that only gross approximations were possible, and the data do not exclude the possibility that there is no ketogenesis at all in tumor cells. An interesting difference was observed between these experiments with mouse liver and previous ones with rat liver slices (13). Whereas in rat liver slices a great pre-

TABLE 3

OXIDATION OF BUTYRATE-1-C^{14} BY LIVER AND NEOPLASTIC TISSUES

Conditions similar to those described in Table 2

	O_2 uptake	Respiratory CO_2		
			R.S.A.	C.C.
	μM	μM	Per cent	μ atoms C
Normal liver	341	176	33.8	59.5
Host liver	428	197	41.9	82.5
Hepatoma 98/15	260	154	33.3	51.3
Host liver	392	148	36.0	53.5
Mammary adenocarcinoma	235	127	9.4	12.0
Host liver	402	146	36.8	53.8
Sarcoma 37	171	132	7.4	9.8
Host liver	333	142	21.5	30.6
Rhabdomyosarcoma	159	122	13.0	15.8

TABLE 4

OXIDATION OF OCTANOATE-1-C^{14} BY LIVER AND NEOPLASTIC TISSUES

Conditions similar to those described in Table 1

	O_2 uptake	Respiratory CO_2		
			R.S.A.	C.C.
	μM	μM	Per cent	μ atoms C
Normal liver	225	137	31.6	43.3
Host liver	394	203	51.1	104
Hepatoma 98/15	221	182	24.7	45.0
Host liver	391	182	49.3	89.8
Mammary adenocarcinoma	50.4	61.8	~1.0	~0.6
Host liver	377	156	40.2	62.7
Sarcoma 37	75.0	80.0	~0.2	~0.2
Host liver	378	221	32.7	72.4
Rhabdomyosarcoma	66.3	113	~0.5	~0.6

TABLE 5

OXIDATION OF PALMITATE-1-C^{14} BY LIVER AND NEOPLASTIC TISSUES

Conditions similar to those described in Table 1. Substrate concentration, 0.0005 M.

	O_2 uptake	Respiratory CO_2		
			R.S.A.	C.C.
	μM	μM	Per cent	μ atoms C
Normal liver	298	228	4.9	11.1
Host liver	314	261	6.6	17.2
Hepatoma 98/15	297	207	5.5	11.4
Host liver	432	289	5.5	15.9
Mammary adenocarcinoma	197	174	3.1	5.4
Host liver	371	289	4.5	13.1
Sarcoma 37	180	173	5.0	8.7
Host liver	371	288	5.2	15.0
Rhabdomyosarcoma	225	241	4.4	10.7

TABLE 6

EFFECT OF OCTANOATE-1-C^{14} CONCENTRATION ON OXIDATIVE CAPACITY OF NEOPLASTIC TISSUE

Conditions similar to those described in Table 1. Substrate concentration varied as indicated.

	Concentration	O_2 uptake	Respiratory CO_2		
			μM	R.S.A.	C.C.
Normal liver	0.01 M	225	137	31.6	43.0
Hepatoma	0.01 M	221	182	24.7	45.0
Mammary adenocarcinoma	0.01 M	50.4	61.8	~1.0	~0.6
"	0.001 M	136	164	6.1	9.9
Sarcoma 37	0.01 M	75.0	80.8	~0.2	~0.2
" "	0.001 M	265	256	11.4	29.2
Rhabdomyosarcoma	0.01 M	66.3	113	~0.5	~0.6
"	0.001 M	206	194	10.6	20.6

ponderance of activity was observed in the carboxyl of acetoacetate over the β-carbon, in these experiments with mouse liver the ratios were more nearly equal but still favored the carboxyl carbon.

"Trapping" of active acetoacetate.—It is well established that, in general, nonhepatic tissues can oxidize acetoacetate readily, and this probably accounts in part for the lack of its accumulation in such organs (9). Inasmuch as our preliminary experiments (Table 1) demonstrated acetoacetate oxidation in tumor cells, the possibility had to be considered that acetoacetate was being produced by tumor cells, but was being rapidly metabolized. To investigate this possibility, a "trapping" procedure was employed in which the isotopic butyrate was oxidized in the presence of a pool of unlabeled acetoacetate. Under these conditions it was expected that any labeled metabolic acetoacetate would mix with the unlabeled material and would be detected by radioactivity of the recovered acetoacetate. As shown in the last two experiments of Table 7, no appreciable activity was trapped by this procedure, and therefore the conclusion seems warranted that ketogenesis is of low or questionable occurrence in the tumor cell. Lack of ketogenesis in the hepatoma is of particular interest in that it represents a marked deviation from the metabolism of the normal liver cell.

TABLE 7

CONVERSION OF BUTYRATE-1-C^{14} TO ACETOACETATE

Conditions similar to those described in Table 1. In experiments 1 to 5, 100 μM of acetoacetate were added as carrier to the suspending medium for the liver slices at the close of the experiment, and 200 μM added to the medium for the tumor slices. In experiments 6 and 7, 300 and 200 μM, respectively, were added to the suspending medium at the start of the experiment and no carrier added afterward.

Exp. no.	Tissue	Resp. CO_2 C.C. μ atoms	Acetoacetate Total C.C. μ atoms	Acetoacetate β C.C. μ atoms	Acetoacetate –COOH C.C. μ atoms
1	Normal liver	59.5	122	56	66
2	Host liver	82.5	88	36	52
	Hepatoma	51.3	4.4	0.4	4
3	Host liver	53.3	125	55	70
	Mammary adenocarcinoma	12.0	3	1	2
4	Host liver	53.8			
	Sarcoma 37	9.8	1	0	1
5	Tumor liver	30.6	90	46	44
	Rhabdomyosarcoma	15.8	6	1	5
6	Host liver, rat	159	152	58	94
	Hepatoma, rat	19.3	5	1	4
7	Host liver, rat	56.0	164	47	117
	Hepatoma, rat	14.2	5	2	3

Oxidation of acetoacetate in liver and tumor slices.—Since tumors appeared to display a nonhepatic metabolic pattern with respect to ketogenesis, it was of further interest to compare the rates at which these tissues oxidize acetoacetate. From the preliminary experiments recorded in Table 1, it is evident that acetoacetate is oxidized in tumor slices at rates at least as rapid as those shown by liver slices. An uncertain feature of these experiments is that they were carried out with carboxyl- and β-labeled acetoacetate. Acetoacetate undergoes a spontaneous decomposition which results in a higher apparent CO_2 formation (12); and, though the data of Table 1 have been corrected for this spontaneous reaction, the accuracy of the correction still remains somewhat doubtful.

TABLE 8

OXIDATION OF ACETOACETATE-2,4-C^{14} BY LIVER AND NEOPLASTIC TISSUES

Conditions similar to those described in Table 1. Substrate concentration, 0.002 M.

	O_2 uptake μM	Respiratory CO_2 μM	Respiratory CO_2 R.S.A. Per cent	Respiratory CO_2 C.C. μ atoms
Host liver	455	371	7.2	26.8
Hepatoma	364	340	16.2	55.1
Host liver	482	319	5.7	18.1
Mammary adenocarcinoma	136	155	13.0	20.2
Host liver	392	312	5.6	17.5
Sarcoma 37	247	216	15.2	32.8
Host liver	382	268	3.3	8.8
Rhabdomyosarcoma	219	170	12.0	20.4
Host liver	349	287	6.3	18.2
Hepatoma, rat	132	161	7.8	12.6
Host liver, rat	272	310	2.2	6.7
Hepatoma, rat	142	165	8.1	13.3
Normal mouse liver	355	261	7.8	20.4

To avoid this source of error and to obtain a better idea of acetoacetate oxidation in tumor cells, further experiments were conducted with acetoacetate labeled in the α and γ carbons. The results, as shown in Table 8, leave no doubt that tumor slices can oxidize acetoacetate. A considerable range of variation in the ability to oxidize acetoacetate was displayed by liver slices, the C.C. values ranging from 6.7 to 26.8. In every experiment except one, however, higher values were observed for the tumor slices. This is particularly significant, since in every instance the over-all respiratory rate, as shown in columns 2 and 3, was considerably lower in the tumor than in the liver slices. This may be more clearly seen in a comparison of the respective R.S.A.'s (column 4). The respiratory CO_2 samples from the tumor slices contained much more acetoacetate carbon than did the CO_2 derived from the liver slices.

Here again, tumor cells, including the hepatoma, displayed a metabolic pattern which resembles that of nonhepatic rather than liver cells.

The results of this investigation leave no doubt that neoplastic cells can carry out the oxidation of fatty acids to CO_2, sharing this property with hepatic and nonhepatic normal cells. Though the results with short-chain acids provide some justification for the belief that oxidation of fatty acids may not proceed as readily in tumor cells, such a conclusion is probably unwarranted in view of various uncertainties in the *in vitro* experiments. It is noteworthy that, with respect to acetoacetate formation and utilization, the neoplastic transformation of the liver cell is associated with a metabolic transformation to a pattern characteristic of nonhepatic tissue. The loss in the ability of the neoplastic liver cell to produce acetoacetate from fatty acids is in line with many examples of the loss of specific metabolic function when normal cells become neoplastic (6). It is of particular interest in this connection that the neoplastic transformation of the liver cell is associated also with an enhancement in a metabolic function—namely, acetoacetate oxidation.

SUMMARY

Slices of transplanted mouse and rat tumors oxidized C^{14}-labeled long- and short-chain fatty acids to CO_2, though at rates generally lower than liver slices. Differences between liver and tumor slices were greater with the short-chain fatty acids, and these differences could be at least partly attributed to a greater inhibitory action of the short-chain acid on the tumor cells.

Fatty acid oxidation in tumors, including hepatoma, resembled that of nonhepatic tissues in that it was not associated with ketogenesis. Tumor slices, including hepatoma, also resembled nonhepatic tissue in having a high capacity for acetoacetate oxidation.

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The Macromolecular Particles of Normal and Regenerating Rat Liver*†

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In the course of an investigation of the various nucleoprotein fractions of mouse spleen it was found that the submicroscopic particle fraction of the cytoplasm showed a number of well defined boundaries in the analytical ultracentrifuge with sedimentation constants ranging from 67 to 30 S (Svedbergs) (11). Since the particles which gave rise to these boundaries were much smaller than the material generally termed "microsomes," the term "macromolecules" seemed more appropriate. In normal mouse spleen the total amount of these substances was about 3 mg/gm of tissue, while in leukemic spleen as much as 7 mg/gm was found. Since the increases observed in the slowly growing spontaneous leukemia were quite similar to those found in rapidly growing transplanted leukemia, they could not be ascribed solely to rapid growth. It therefore seemed worth while to analyze other types of tissue, including rapidly growing and neoplastic. In this paper analyses of normal and regenerating rat liver are reported. A similar study on azo dye-induced rat liver tumors will be described elsewhere.

MATERIALS AND METHODS

Adult rats of the Wistar strain, weighing from 180 to 300 gm., were used. They were maintained on Purina chow ad libitum up to the time of sacrifice. A group of three rats was used for each experiment, except in two cases where animals were lost through postoperative death.

Partial hepatectomies were performed under ether anesthesia by the method of Higgins and Anderson (6). At periods of 1–12 days after the operation the rats were anesthetized with ether just long enough for the intraperitoneal injection of 0.5 ml. of a solution containing 15 mg. of nembutal and 2.5 mg. of heparin. After the liver had been perfused (by injecting saline into the dorsal aorta and cutting the hepatic portal vein), it was removed, chilled on ice, and weighed.

The cold liver was then forced through a stainless steel mincer with holes $\frac{3}{4}$ mm. in diameter, and the resulting pulp was weighed and homogenized in 0.88 M sucrose as previously described (11). Samples of the whole homogenate were taken for smears and nuclear counts, and the remainder was fractionated by differential centrifugation. Tissue debris, whole cells, nuclei, and mitochondria were removed by centrifuging for 30 minutes at 20,000 × *g*, and the particles were concentrated by sedimentation for 2 hours at 152,000 × *g*. The details of the procedure have been given elsewhere (11). The particles were resuspended in veronal-chloride buffer, pH 8.6 and ionic strength 0.10, to a volume of 1.25 ml/gm of original liver pulp, and analyzed immediately in the ultracentrifuge[1] at 96,000 × *g*.

The amounts of the various components were estimated from the areas under the sedimentation boundaries (11), and converted to mg/gm of liver pulp. No correction was made for the percentage of liver cells not broken, since they amounted to less than 10 per cent.

Smears of the whole homogenate were made on albuminized slides and fixed in acetic acid-alcohol. They were stained with hematoxylin and eosin or with aceto-orcein-fast-green (8), and the percentage of broken cells was determined by differential counts. At first 500 nuclei and cells were counted on each of six slides. After 500 were found to be representative of the whole homogenate, that number was counted on only one slide of each type of stain.

Total nuclear counts were made in a hemocytometer by the procedure of Price and associates (13). Ten counts were averaged for each homogenate; the probable error of the mean was 1–4 per cent. In a number of experiments the percentage of broken cells found in the hemocytometer agreed within 7 per cent with the value found on the smears.

Total nitrogen was determined by semi-micro-Kjeldahl analysis.

RESULTS

The ultracentrifuge patterns found for rat liver particles differ from the mouse spleen patterns (11) in several respects. While some of these differences may be ascribed to the use of more concentrated particle suspensions in the present work, there seem to be real differences between the two types of tissue. Characteristic patterns for normal and regenerating rat liver are shown in Figure 1. In the normal liver a definite microsome boundary (M) appears before the centrifuge has reached full speed (Fig. 1, left-hand column). Estimation of the area under this boundary was difficult because of its rapid spreading and the turbidity which ac-

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[1] Specialized Instruments Corporation, Belmont, Calif.

companied it. Its sedimentation rate was about 130 S. Following the microsomes there are six macromolecule boundaries, which have been lettered for convenience. These can be seen most clearly in the patterns of regenerating liver. Their average sedimentation rates were: A″, 107 S; A′, 90 S; A, 72 S; B, 49 S; C, 38 S; and E, 27 S. The two fastest boundaries, A″ and A′, have never been observed in mouse spleen, and A has been seen only a few times. The B boundary of liver apparently corresponds to the B boundary in spleen; recent analyses of leukemic mouse liver (a mixture of hepatic cells and leukemic white cells) have shown a B boundary just as sharp as that seen in either normal liver or spleen. The lower sedimentation rates found for B (and all the other boundaries) in the rat liver experiments can be ascribed to the higher concentrations of both protein and sucrose in these preparations. The C and E boundaries of liver, like those of spleen, sediment about 80 and 55 per cent as fast as the B boundary, but unlike spleen there is no indication of any D boundary in the liver. As in the case of the spleen particles (11), there always appears to be a large boundary near the meniscus. This is caused by a small slow boundary (S_{20} about 3 S), superimposed on a turned-up base-line. No attempt has been made to estimate the amount of slow-moving material, since the degree of upturn in the base-line is dependent on the amount of sucrose resuspended with the pellets and is difficult to reproduce in reference base-line experiments. In normal rat liver the characteristic pattern shows a fairly definite microsome boundary and five or six macromolecule boundaries, with boundary B predominating (Fig. 1, top row). Two days after partial hepatectomy (Fig. 1, bottom row) the pattern shows a marked decrease in the concentrations of the microsomes and of component B, and equally striking elevations in components C and E.

Since components A″ and A′ are present only in small amounts (about 0.1 and 0.3 mg/gm of liver) and show no consistent changes during the regenerative process, they will not be reported in detail. The concentrations of microsomes and of components A, B, C, and E found in normal liver and at definite periods after partial hepatectomy are shown in Chart 1, together with the number of cells per gram of liver. On the first day following partial hepatectomy the concentration of microsomes drops sharply. It then rises gradually as regeneration proceeds, but is still well below the normal level on the 12th day. The concentration of component A also drops on the first day and remains low throughout the period studied.

Component B was the most variable of any of the substances measured. The average value shows a decrease which is maintained from the 2d to the 7th days, while the 12th-day value has returned to the normal level.

The changes observed in component C are in just the opposite direction. While, in normal liver, the concentration of C is about 0.8 mg/gm, on the

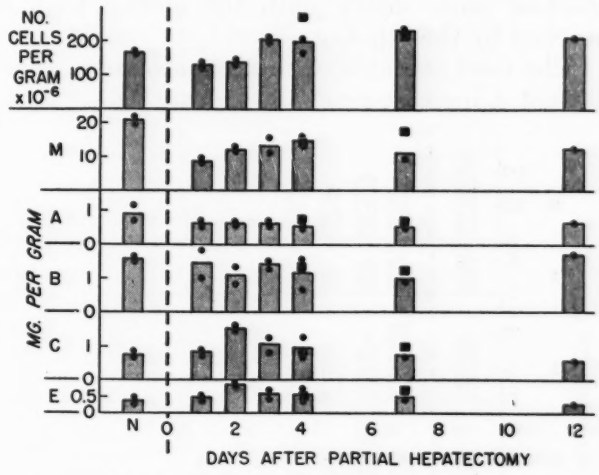


CHART 1.—The number of cells and the concentration of microsomes, M, and macromolecular components, A, B, C, and E, per gram of liver pulp. ● = pool of three livers; ■ = one liver only.

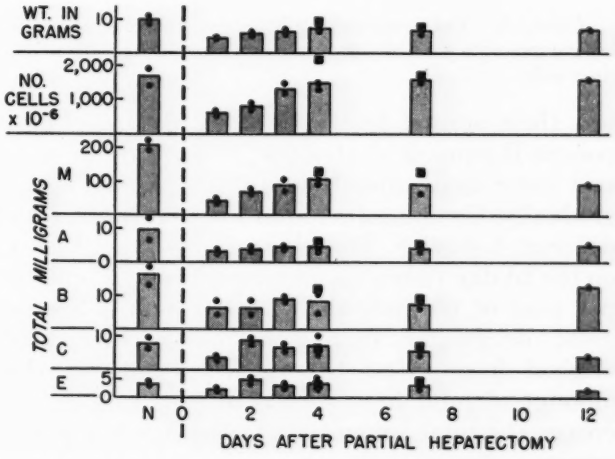


CHART 2.—The average liver weight, total number of cells, and total amounts of microsomes and macromolecular components per liver. ● = pool of three livers; ■ = one liver only.

2d day it has risen to nearly double its original value. Following this, the values gradually fall again, until the normal level is reached by the 7th day. The behavior of component E closely parallels that of C.

The total nitrogen content of the particles was about 4.4. mg/gm of normal liver. It dropped to 3.6 mg/gm on the 1st day of regeneration and remained at this level through the 12th day.

In Chart 2 the average liver weight, the total

number of cells, and the total amounts per liver of the five cytoplasmic components are given. The liver weight increases fairly rapidly up to the 4th day, then levels off at about 80 per cent of the normal value. The number of cells shows some increase between the 1st and 2d days, rises sharply between the 2d and 3d days, and then continues to increase more slowly until the normal level is reached by the 7th day.

The total amounts of microsomes and of component A increase gradually but are still well be-

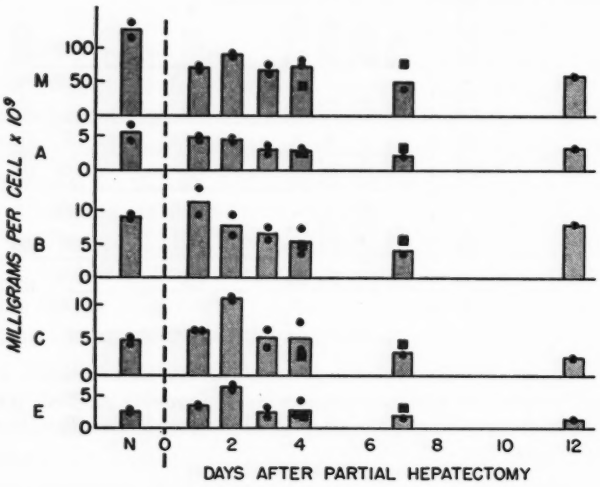


CHART 3.—The amounts of microsomes and macromolecular components per cell. ● = pool of three livers; ■ = one liver only.

low their normal levels on the 12th day. Component B remains at about 40 per cent of the normal value until after the 2d day, then increases gradually. Components C and E again show a very different behavior. They increase so rapidly that on the 2d day (when the liver weight is still only 60 per cent of normal) their total amounts are as great, or greater, than are found in the whole original liver. After the 2d day, although the amount of total liver substance continues to increase, the total amounts of C and E actually decrease.

The amounts of microsomes and of components A, B, C, and E, calculated on a per cell basis, as suggested by Price and Laird (12), are shown in Chart 3. Here, changes in the levels of the various components reflect their abilities to keep pace with cell division; thus, every component shows a drop between the 2d and 3d days, when cell division is proceeding the most rapidly. Components C and E again show a pronounced elevation on the 2d day.

DISCUSSION

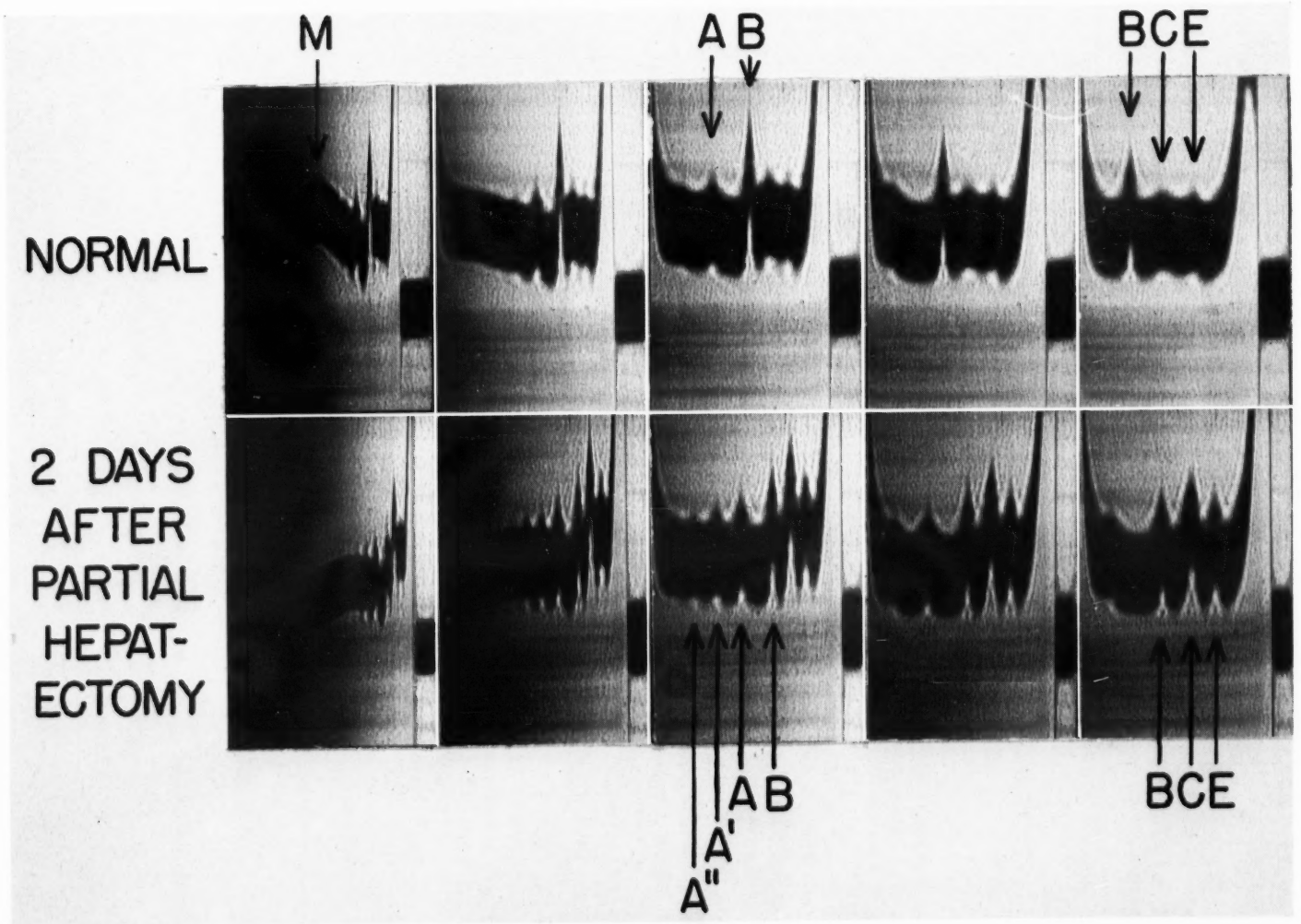
The general course of the regenerative process agreed closely with the observations of others (2–4, 6, 9, 12, 14), except that the decreases in total particle nitrogen and in microsomes per cell found on the 1st day differ from the finding of Price and Laird (12), who observed an initial rise in the nitrogen content of the "small granules." The two preparations are not closely comparable, however, since their "small granule" fraction[2] was made up of particles sedimenting between 10 minutes at 19,000 × *g* and 36 minutes at 120,000 × *g*, while our "particle" fraction includes that part of the material which sediments between 30 minutes at 20,000 × *g* and 120 minutes at 152,000 × *g*.

While there was some increase in the total number of cells between the 1st and 2d days, the greatest increase took place between the 2d and 3d days. The greatest change in the relative amounts of components B and C was found on the 2d day, just preceding the period of most rapid cell division; the ratio of C to B, which was 0.49 in normal liver, had risen to 1.38. By the 3d day, when the rate of cell division was declining, the ratio had returned to 0.76. It remained at about this level through the 7th day, but had fallen to 0.32 by the 12th day. The parallelism between the ratio of C to B and the rate of cell division throughout the entire period studied is striking.

Since the earlier analyses on mouse spleen were made in a more dilute solution, where the C, D, and E boundaries of normal spleen could not be clearly differentiated, no similar comparison with leukemic spleen could have been made (11). The sedimentation analysis of normal mouse spleen particles was therefore repeated at a higher concentration, where the individual boundaries could be distinguished. The ratio of C to B was found to be only 0.20, much lower than the average value for the spleens of mice with spontaneous leukemia, 0.33, or that for the spleens of mice with trans-

[2] J. M. Price and A. K. Laird, personal communication.

FIG. 1.—Sedimentation patterns of rat liver particles: normal and two days after partial hepatectomy. The direction of sedimentation is to the left. Pictures taken after 2, 6, 10, 14, and 18 minutes at 37,020 r.p.m.


M
A B
B C E
NORMAL
2 DAYS AFTER PARTIAL HEPAT-ECTOMY
A B
A'
A"
B C E

planted leukemia, 0.39. While an explanation of these observations awaits further work, some correlation between either the relative or the absolute amounts of the smaller particles, C and E, and the rate of cell division is definitely indicated.

The pentosenucleic acid (PNA) content of the macromolecular particles was not determined, since no direct measurements can be made until they have been separated from the microsomes and the slowly sedimenting substance which always accompany them in these preparations. There is much indirect evidence, however, to suggest that they are rich in PNA. When differential centrifugation of liver cytoplasm is carried out in salt solutions, the fractions obtained at the highest centrifugal forces are always the richest in PNA (1, 5, 7). Similar results have been obtained in this laboratory on spleen cytoplasm centrifuged in 0.88 M sucrose. The larger submicroscopic particles (sedimented in 4 hours at 20,000 × *g* from 0.44 M sucrose) had PNA to nitrogen ratios of only 0.3–0.6 (10); but when the centrifugal force was strong enough to sediment 90 per cent of the PNA (3 hours at 180,000 × *g* in 0.88 M sucrose) PNA to nitrogen ratios as high as 1.8 were found for particles from leukemic spleen.[3]

SUMMARY

The macromolecular particles, presumably nucleoprotein, from the cytoplasm of normal and regenerating rat liver have been studied in the analytical ultracentrifuge. Seven boundaries were seen, with sedimentation rates of 130, 107, 90, 72, 49, 38, and 27 S, corresponding to the microsomes and six macromolecular components. Regenerating liver showed a marked drop in the concentrations of the faster components accompanied by a rise in the two slowest components, especially during the period of most rapid cell division.

[3] M. L. Petermann and A. M. Larack, unpublished data.

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The Age of the Tumor-bearing Hosts as a Factor Conditioning the Transmissibility of the Rous Sarcoma by Filtrates and Cells*†

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Fundamentally, the present paper is concerned with both the state of the Rous sarcoma virus—masked or free—when the tumor is made to grow either in old or in young hosts, as well as with properties of the tumor itself, associated with the virus in either state.

It is well known that the causative viruses of chicken sarcoma can vanish in the most unpredictable way, so that filtrates from apparently typical tumors become absolutely and persistently negative. The best example is that of Gye and Andrewes (14), who failed to show active virus in filtrates and desiccates of the Rous sarcoma in nine successive cell passages. Among studies concerning the conditions governing this phenomenon are the findings of Doerr *et al* (7) and of Carr (6), showing that the activity of filtrates of the Rous sarcoma decreases to complete inactivity as the tumors grow older. The influence of the age of the host was reinvestigated by one of us (10) in a study of fourteen spontaneous chicken sarcomas in which only one out of four tumors occurring in hosts 12–18 months old was transmissible by cells and filtrates, whereas at least four out of ten tumors occurring in younger hosts were transmissible by the same preparations.[1]

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† The experimental material of this paper has been included in a dissertation presented by the junior author to the Faculty of Yale University School of Medicine, in partial fulfilment of the requirements for the degree of Doctor of Public Health.

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[1] In this paper the terms "filtrability" and "nonfiltrability" will be used for convenience and not to indicate that the inactivity of the filtrates is due to the incapacity of the virus to pass through filters. The same fluctuations in activity have been observed with tumor preparations in which the cells have been eliminated or killed by means other than filtration (see Carr [6] and Miszurski *et al* [18]).

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MATERIALS AND METHODS

The animals studied were 44 adult chickens and 38 chicks, of the Plymoth Rock breed, which were at an average age of 15.4 months and 16.0 days, respectively. These birds are designated "original hosts," and their tumors "original tumors."

The Rous sarcoma was maintained by cell passages through chicks. Of the 82 tumors studied, 48 were induced by filtrates and 34 by cell suspensions. Both materials were injected into the breast in varying amounts, but the effects of variation in the amount of inoculum is not a factor in the present investigation. Chickens of the two age groups were inoculated at the same time with the same or comparable tumor materials.

The birds were killed at different intervals so as to provide tumors of different ages, and representative portions of the growths were secured for processing and for histological study.

From each tumor a cell suspension was prepared with 1 part of minced tumor tissue plus 4 of saline, and 0.1 cc. of the material was injected subcutaneously in each side of the breast of four chicks. Filtrates were prepared by grinding 1 part of tumor with sand and 19 volumes of saline, centrifuging the mixture, and passing the supernatant fluid through Berkefeld N candles. The filtrates were further diluted with saline, so that dilutions at 1:20, 1:200, 1:2,000, and 1:20,000 were obtained. Each filtrate was inoculated into six or more chicks, each of which was given an intradermal injection, in both sides of the breast and the outer side of the thighs, of 0.5 cc. of each of the four different dilutions. The sites of inoculation of each of the dilutions were shifted in different chicks.

The areas of the tumors that developed (designated as "test tumors") were measured and recorded, generally 30 days after injection, when the birds were killed, or following natural death. In most cases the chicks developed well delimited tumors at the sites of inoculation, and whenever the growths merged with each other the results of the corresponding inoculations were discarded. Data from chicks dying within 10 days after inoculation were not included in the results. Autopsies were conducted in every case.[2]

Transmissibility of the Tumors by Filtrates

Considering the 82 original tumors with respect to the activity or inactivity of their filtrates after inoculation into the test chicks, the results have been presented (Table 1) according to the age of the original hosts, the age of the original tumors, and the source material—cell suspensions or filtrates—by which the original tumors were induced. From these results, three main effects are

[2] The statistical analysis of the results was carried out by one of us (P. M. Freire) and can be found in detail in his thesis for the degree of Doctor of Public Health. The authors are much indebted to Doctor C. I. Bliss for valuable help in this regard.

apparent: (*a*) 32 out of 38 tumors (84.2 per cent) from young chickens were filtrable as against seventeen out of 44 tumors (38.6 per cent) from adult chickens; (*b*) as best shown in the last column of the table, an increase in the age of the tumors corresponded to a decrease in filtrability; and (*c*) 26 out of 34 tumors induced by cell suspensions (76.5 per cent) were filtrable, as against 23 out of 48 tumors (47.9 per cent) induced by filtrates.

Since each tumor tested was under the influence of three variables—age of the host, age of the tumor, and source material, it was necessary to evaluate the significance of each effect by a method that took into account the concomitance of these variables. It seemed appropriate to utilize the methods developed by Bliss (1–4) for the calculation of dosage-effect curves. Each group of the hosts was considered as providing two separate curves, one corresponding to original tumors induced by cell suspensions and the other corresponding to original tumors induced by filtrates. Therefore, each calculated regression line described, as an all-or-none response (positive or negative filtrates), the effect of the varying age of the original tumors.

The results of the analysis proved the statistical significance of the effects of age of the host, age of the tumor, and source material on the filtrability of the tumors, and the probabilities that the results were due to chance alone were 1 in 1,000, 1 in 170, and 1 in 500 for each of the three effects, respectively.

From this study it may be concluded: first, that the filtrability of the tumors, which is actually the presence in them of virus in a free state, is in an inverse relation to the age of the host and the age of the tumor; and, second, that free virus is more frequently present in the tumors induced by cell suspensions than in those induced by filtrates.

TABLE 1

FILTRABILITY OF TUMORS IN RELATION TO AGE OF THE HOSTS, AGE OF THE TUMORS, AND SOURCE MATERIAL

Age group of the original hosts	Age group of the original tumors (days)	Original tumors induced by cell suspensions: Age in days Geometric mean	Original tumors induced by cell suspensions: Proportion with positive filtrates	Original tumors induced by cell suspensions: Per cent	Original tumors induced by filtrates: Age in days Geometric mean	Original tumors induced by filtrates: Proportion with positive filtrates	Original tumors induced by filtrates: Per cent	All source materials: Tumor age, in days Geometric mean	All source materials: Proportion of tumors with positive filtrates	All source materials: Per cent
Young	≦20	16.6	9/9	100.0	19.1	6/6	100.0	17.6	15/15	100.0
	21–40	22.0	2/2	100.0	28.9	7/9	77.8	27.5	9/11	81.8
	>40	50.7	6/7	85.7	51.3	2/5	40.0	50.9	8/12	66.7
	All ages	26.5	17/18	94.4	29.4	15/20	75.0	28.0	32/38	84.2
Adult	≦30	23.2	2/3	66.7	21.8	4/10	40.0	22.1	6/13	46.2
	31–40	31.3	3/3	100.0	34.4	3/9	33.3	33.6	6/12	50.0
	41–60	45.9	3/5	60.0	46.9	1/5	20.0	46.3	4/10	40.0
	>60	75.7	1/5	20.0	79.6	0/4	0	77.4	1/9	11.1
	All ages	44.0	9/16	56.3	34.8	8/28	28.6	37.9	17/44	38.6
All host and tumor ages		33.7	26/34	76.5	32.5	23/48	47.9	33.0	49/82	59.8

Appropriate calculations showed that, as pointed out by a nonsignificant χ^2 in young as well as in adult chickens, there was no association between the degree of activity of the positive filtrates and the age of the tumors. Disregarding the differences in tumor age the effects of the age of the hosts and that of filtrates or cell suspensions were evaluated: the corresponding χ^2 analyses were again nonsignificant.

The above results were supplemented by data from other experiments in which 29 unfiltered tumor extracts were titrated in chicks in much the same manner as described for filtrates. Each extract was prepared from pooled material from three tumors induced by cell suspension and grown for 9–16 days in chicks 12–21 days old at the moment of inoculation. The results showed that, despite the uniformity of the age of the host,

DEGREE OF ACTIVITY OF THE FILTRATES

We next studied whether the factors that influence filtrability as a qualitative process also influenced the degree of activity of the positive filtrates. Since there was only a rough parallelism between the areas of tumors or their logarithms and the doses of filtrate, the data were interpreted in terms of an all-or-none response—the presence of a tumor, regardless of its size, being considered as a positive response. To this effect, 50 per cent end-points were calculated by the method of Reed and Muench (19). The positive tumor filtrates were classified in three degrees of activity, according to the following median effective doses, in miligrams of tumor tissue: high titer < 0.079; intermediate titer, from 0.0079 to 7.9; low titer > 7.9.

age of the tumor, and source material, there was such a variation in the titers of the extracts that some of them were nearly 100 times more active than others.

In summary, the three factors that were found to condition the filtrability of the tumors—namely, age of the hosts, age of the tumors, and source material used in inducing the tumors—did not appear to have any significant influence upon the titers of the filtered or unfiltered extracts of the tumors. These results are apparently at variance with those of Carr (6), who found the virus titer to be inversely proportional to the age of the tumors. An explanation of the discrepancy can be the fact that, contrary to us, Carr used highly inbred strains of chickens.

Reversion of the Masked Virus to a Free State

The possibility that the virus was present in the nonfiltrable tumors in a changed or masked form was considered. Passage of the tumors by cell suspensions into other hosts could be a means of revealing its presence. This was done with eight of our 33 nonfiltrable tumors. Six of these tumors had grown in adult hosts and two in young hosts. Of the adult-grown tumors, two were passed into other adults and four into young hosts. The two tumors grown in young hosts were passed into other young hosts. The results were clear-cut: all the resulting tumors yielded active filtrates when tested in the usual way. Evidently, no masking effect took place in the new hosts, either adult or young, and the virus reverted to a free, active form in all cases.

This reversion of the virus was further verified in a number of chicks inoculated with another nine of the 33 nonfiltrable sarcomas. The resulting tumors in these chicks were not tested for filtrability, but the occurrence of secondary lesions of the non-neoplastic, hemorrhagic, cell-destructive type was ample evidence of the presence of a free virus.

In summary, by transplantation of nonfiltrable tumors into other hosts, the virus reverted from a masked to a free state, as shown either by the induction of hemorrhagic lesions or by the activity of filtrates from the growths induced by the nonfiltrable original tumors.

Transmissibility of the Tumors by Cell Suspensions

The majority of the original tumors proved to be transmissible by inoculation of cell suspensions into 2-week-old chicks. Only three tumors—64, 82, and 84 days old—which had been grown in 1-year-old chickens and gave inactive filtrates, could not be transmitted by cells. The transmissible tumors gave different proportions of takes in the test chicks. The results of these tests, pooled for different classes of tumors, are presented in Table 2, where the original tumors are classified according to the age of the host, age of the tumor, and filtrability.

TABLE 2

INCIDENCE OF TUMORS IN TEST CHICKS INOCULATED WITH CELL SUSPENSIONS

Age group of the original hosts	Age group of the original tumors (days)	Filtrable original tumors			Nonfiltrable original tumors			Filtrable and nonfiltrable original tumors		
		Age in days Geometric mean	Proportion of test chicks developing tumors	Per cent	Age in days Geometric mean	Proportion of test chicks developing tumors	Per cent	Age in days Geometric mean	Proportion of test chicks developing tumors	Per cent
Young	≦20	18.9	31/32	96.9				18.9	31/32	96.9
	21–40	29.2	32/35	91.4	21.0	8/8	100.0	27.5	40/43	93.0
	>40	50.0	28/32	87.5	52.8	9/11	81.8	50.9	37/43	86.1
	All ages	29.6	91/99	91.9	38.8	17/19	89.5	31.2	108/118	91.5
Adult	≦30	24.3	24/25	96.0	20.5	16/24	66.7	22.1	40/49	81.6
	31–40	33.3	17/21	81.0	33.8	21/26	80.8	33.6	38/47	80.9
	41–60	46.7	13/15	86.7	46.1	14/20	70.0	46.3	27/35	77.1
	>60	83.9	4/4	100.0	76.6	14/30	46.7	77.4	18/34	52.9
	All ages	34.0	58/65	89.2	40.6	65/100	65.0	37.9	123/165	74.6
All host and tumor ages		31.3	149/164	90.9	40.3	82/119	68.9	34.9	231/283	81.6

In order to ascertain the degree and significance of the influences exerted by these three factors and by the source material of the original tumors upon their transmissibility by cells, a similar method of statistical analysis was used as in the study of transmissibility by filtrates. The analysis showed the following: (*a*) tumors induced by filtrates did not differ significantly in transmissibility from tumors induced by cells; (*b*) older tumors were significantly less transmissible than younger ones; (*c*) filtrable tumors grown in adult chickens did not differ significantly in transmissibility from filtrable tumors grown in young chickens; (*d*) nonfiltrable tumors grown in adult chickens were significantly less transmissible than nonfiltrable tumors grown in young chickens; (*e*) nonfiltrable

tumors grown in young chickens did not differ significantly from filtrable tumors grown in young chickens; and (*f*) nonfiltrable tumors grown in adult chickens were significantly less transmissible than filtrable tumors grown in adult chickens.

In brief, there was a significant decrease in the transmissibility of tumors by cell suspensions—first, with an increase in the age of the tumor and, second, for tumors of the same age in adult chickens, with the absence of free virus as manifested by nonfiltrability.

Incidence of Metastases in the Original Hosts

Among 80 chickens of whose tumors the filtrability was studied, 31 (38.8 per cent) had visceral metastases: 25 in the lungs, seven in the liver, three in the heart, and one in the ovary.

In Table 3 the incidence of metastases is shown in relation to the age of the hosts, age of the tumors, and source material which induced the tumors. According to the raw figures, the tumors induced by cell suspensions as well as those induced by filtrates gave more metastases when grown in young chickens than when grown in adult chickens. Also, in young as well as in adult chickens, the tumors induced by cell suspensions metastasized more often than those induced by filtrates. Moreover, a lower incidence of metastases was observed, in general, when the primary tumors had grown for a longer period before the hosts were killed—older tumors, as contrasted with young tumors.

The inverse relation between the incidence of metastases in the original hosts and the age of the primary tumors in the same hosts was probably only apparent, since one cannot assume that the metastases of an early age disappeared as the tumors grew older. This point will be considered in a following paper. In order to evaluate the significance of the effects of age of the hosts and source material of the tumors upon their metastasizing power, the relation between tumor age and incidence of metastases had to be taken into account in the statistical analysis.

Dose-response curves were calculated with data from Table 3 in the same way as in the study of transmissibility by filtrates, described above. The combined slope of the calculated regression lines was not statistically significant, showing that the age of the tumors was not significantly related to the incidence of metastases. Therefore, the effects of age of the hosts and of source material were tested as a 2×4 contingency table with χ^2 based upon the pooled figures for all tumor ages. This χ^2 ($= 5.153$) was not significant.

TABLE 3

INCIDENCE OF METASTASES IN THE ORIGINAL HOSTS

Age group of the original hosts	Age group of the original tumors (days)	Original tumors induced by cell suspensions: Age in days Geometric mean	Original tumors induced by cell suspensions: Proportion with metastases	Original tumors induced by cell suspensions: Per cent	Original tumors induced by filtrates: Age in days Geometric mean	Original tumors induced by filtrates: Proportion with metastases	Original tumors induced by filtrates: Per cent	All source materials: Tumor age in days Geometric mean	All source materials: Proportion of tumors with metastases	All source materials: Per cent
Young	≦20	17.2	4/7	57.1	19.1	3/6	50.0	18.0	7/13	53.8
	21–40	22.0	2/2	100.0	28.9	3/9	33.3	27.5	5/11	45.5
	>40	50.7	4/7	57.1	51.3	1/5	20.0	50.9	5/12	41.7
	All ages	28.4	10/16	62.5	29.4	7/20	35.0	29.0	17/36	47.2
Adult	≦30	23.2	1/3	33.3	21.8	3/10	30.0	22.1	4/13	30.8
	31–40	31.3	3/3	100.0	34.4	3/9	33.3	33.6	6/12	50.0
	41–60	45.9	2/5	40.0	46.9	2/5	40.0	46.3	4/10	40.0
	>60	75.7	0/5	0	79.6	0/4	0	77.4	0/9	0
	All ages	44.0	6/16	37.5	34.8	8/28	28.6	37.9	14/44	31.8
All host and tumor ages		35.3	16/32	50.0	32.5	15/48	31.3	33.6	31/80	38.8

In brief, neither the age of the host nor the kind of material—filtrate or cell suspension—that induced the original tumors showed statistically significant influences on the incidence of metastases.

On the other hand, the incidence of metastases in the original hosts appeared to be significantly associated with the transmissibility of the respective primary tumors either by filtrates or by cells, as shown in Tables 4 and 5. In other words, the absence of free virus, a lower degree of transmissibility by cells, and the absence of metastases occurred as associated phenomena.

Some Additional Observations on the Tumors Studied

Latent period.—Our data on the latent period of the original tumors are only approximate, since the inoculated animals were usually examined at intervals of 7–10 days. The time that elapsed between the inoculation and the middle of the interval between the last negative and the first positive examinations was taken as the latent period of a tumor. If a growth was already present when the

animal was first checked, the latent period was assumed to be one-half of the interval between the day following the inoculation and the first examination.

In relation to the ages at which the original tumors were tested and for each group of host age, the averages in days of the observed latent periods were as follows: Tumors from young chickens—less than 20 days, 8.1; from 21 to 40 days, 14.0; more than 40 days, 28.3. Tumors from adult chickens—less than 30 days, 10.5; from 31 to 40 days 12.9; from 41 to 60 days, 11.5; more than 60 days, 17.5.

TABLE 4

INCIDENCE OF METASTASES IN THE ORIGINAL HOSTS AND TRANSMISSIBILITY OF THE CORRESPONDING PRIMARY TUMORS BY CELL SUSPENSIONS

Test chicks inoculated with cell suspensions of the original tumors	Metastasizing original tumors	Nonmetastasizing original tumors	Total
Developing tumors	95	136	231
Not developing tumors	9	43	52
Total	104	179	283

$\chi^2 = 9.360$
$P = 0.002$

TABLE 5

INCIDENCE OF METASTASES IN THE ORIGINAL HOSTS AND FILTRABILITY OF THE CORRESPONDING PRIMARY TUMORS

Filtrates	Metastasizing original tumors	Nonmetastasizing original tumors	Total
Positive	27	20	47
Negative	4	29	33
Total	31	49	80

$\chi^2 = 14.926$
$P < 0.001$

Thus, increasing ages of the original tumors corresponded to increasing latent periods, especially in young chickens. On the other hand, the ages of the original tumors were in general inversely related to their speeds of growth, since usually tumors that grew fast were tested earlier in their development, whereas those growing slowly were tested at later ages. Therefore, one may say that, in general, the latent periods of the original tumors were inversely related to the speeds of growth of these tumors before they became noticeable.

Since it was known from previous studies that the dose of the inoculated virus is inversely related to the latent period (5) and directly related to the rate of growth (8), these results add further information concerning the inverse relation existing between content of free virus and age of the tumors.

On the other hand, no significant correlation was found between latent period and age of the host or source material inoculated.

GROSS AND MICROSCOPIC FEATURES OF CHICKEN TUMOR I

Data on these traits of the tumors may prove of interest, because they reveal differences in the intercellular ground substance of the growths (9). By assigning four arbitrary degrees to firmness and to viscidity and by analyzing the results statistically, it was found that firmness of the tumors was directly and significantly correlated with the age of the hosts and, in adult hosts, with the age of the tumors. Viscidity was inversely correlated with the age of the hosts, although the statistical significance was borderline. There was a highly significant negative correlation between firmness and viscidity. In relation to findings on the papilloma virus in wild or domestic rabbits (16), no differences were found in the degree of hemorrhage and necrosis between filtrable and nonfiltrable tumors.

The general histological appearance of the tumors in the adult chickens consisted of packed bundles of elongated fibroblasts frequently associated with an infiltration of lymphoid-like cells, whereas in young chickens the tumors were more pleomorphic, loosely arranged, had large amounts of intercellular matter, and were seldom infiltrated. However, the histological picture was apt to vary much in different areas of the same tumor.

OBSERVATIONS ON OTHER CHICKEN TUMOR STRAINS

In the routine passages of other filtrable chicken sarcomas kept in our laboratory for several years, hosts and tumors of different ages have been used. Two of these tumors, C and E, have been described (10, 13). Statistical analysis of the available results showed that in tumor E and in another filtrable sarcoma not yet described, BV 34, there was a significant negative correlation between the transmissibility by cells and either the age of the tumor—in the case of tumor BV 34—or the age of the host—in the case of tumor E. With tumor C the results were likewise suggestive of an age influence, although not statistically significant.

DISCUSSION

The present study indicates that there is a factor in aging chickens which is responsible for the apparent disappearance (masking) of the causative virus of the Rous sarcoma. Associated with this phenomenon, the transplantability of the tumor by cells and the induction of metastases are very much impaired. The nature of the age factor —virus antibody or other agencies—will not be discussed in the present publication.

Two points, closely related to each other, must first be considered concerning the masking of the virus.[3] The first is whether the change in the virus is of a quantitative or a qualitative order; the second is whether the virus is inactivated in the growing tumor or in the process of preparing the ex tracts.

With the data at hand no definite conclusions can be reached, although the following facts argue that, at least partly, masking is a qualitative phenomenon taking place in the growing tumor:

First, as shown in this study, free virus is more frequently found in extracts of the tumors induced by cells than in extracts of tumors induced by filtrates. Such a fact certainly cannot be explained by events taking place during processing of the tumor, whereas, as we shall discuss later, it could be explained by phenomena operating in the living animal.

The second fact is that in the Rous sarcoma, as in other virus infections, the minimal infective dose is an extremely small amount of virus, probably just one virus particle (9, 12), and, therefore, one such particle present in the filtrate injected would be enough to induce a growth.

The third fact is from a forthcoming study, the results of which must necessarily be here advanced: viruses from old sarcomas that still remained filtrable induced tumors with a significantly lower incidence of metastases than the tumors induced by viruses from young sarcomas. Thus, a qualitative change had occurred in the virus before it became masked in the aging tumor.

The above could lead to the assumption that masking of the virus is the result of a further effect from the aging host acting on the tumor. Then, the effect of the tumor age, as described by others (6, 7) and confirmed in the present investigation, would be a natural consequence of the action of the age factor acting in a cumulative fashion as the age of the tumor increases.

As to the effect of the source material, the virus in filtrates would be exposed to the age factor from the moment of inoculation, whereas the virus associated with cells would be protected at the critical moment of inoculation. This protection would go on during the first stages of formation of the tumor, if we consider that the tumor may get its start from multiplication of the inoculated cells. If the interpretation of these differences is correct, it would carry two implications of interest. The first is that the many generations of virus units derived from those originally present in the filtrates injected kept either a high degree of susceptibility to the age factor or an imprint of the initial masking effect. This would hold true even if one assumes that the virus has been acted upon by immune bodies or other agents during the preparation of the filtrates. The second implication is that the protection afforded the virus by the cell against the masking effect of the age factor is only relative, since tumors induced by cell suspensions also become nonfiltrable in the course of time.

Whatever the nature of the phenomenon, it would seem logical to assume that masking of the virus on the one hand, and impaired activity of the malignant cell such as low transplantability and low metastasizing power on the other hand, are related rather than coincidental phenomena. This would lead to the conclusion that, as discussed elsewhere (10, 11), properties of the malignant cell are conditioned by properties of the causative virus, and that the effect of the age factor on the cell is at least to some extent exerted through an effect on the virus. Whether the age factor can also exert a depressing effect on the malignant cell independently from a virus effect is, of course, a possibility, but suggestive facts in chicken tumors are thus far lacking.

The change induced in the virus by the age factor is only transitory and reversible in nature, for, by the simple expedient of transplanting tumor cells from a nonfiltrable tumor into other hosts, especially young hosts, free virus is easily recovered; conversely, free virus from a young host easily becomes masked in an old host.

These facts may be accepted as a reasonable explanation of the irregularity in the detection of virus in chicken tumors, especially when these are propagated through adult hosts, as is generally the case.

SUMMARY

The filtrability of the Rous sarcoma is inversely related to the age of the hosts and age of the tumors. Also, the tumors induced by cell suspensions have a higher filtrability than those induced by filtrates.

The transmissibility of the tumors by cells is inversely related to the age of tumors and, under certain conditions, directly related to their filtrability.

The occurrence of metastases is directly related to the filtrability of the tumors and to their transmissibility by cells.

Virus can be recovered from the nonfiltrable tumors by passage of the sarcoma cells into other hosts.

[3] There are several points in common between the subject here discussed and that concerned with the masking of the papilloma virus in rabbits. Pertinent papers (15–17, 20, 21) and still others can be consulted in this regard.

The findings are discussed in relation to the effect of the age factor on the tumor and its causative virus.

ADDENDUM

While this paper was in press a study has been published (E. A. Eckert, D. G. Sharp, D. Beard, and J. W. Beard, J. Nat. Cancer Inst., **13**:533–42, 1952) showing that the infectiousness of the virus of erythromyeloblastic leukosis is inversely related to the length of the interval between the inoculation and the bleeding of the donor bird. The analogy between those results and these here presented is pointed out by the authors: This "brings erythromyeloblastic leukosis into line with the chicken sarcoma and rabbit papillomatosis with respect to the occurrence of masked virus."

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The Development of Metastases from the Rous Sarcoma in Relation to Some Characteristics of Its Causative Virus*†

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It was shown in previous studies that both increased age of the host (10) and increased age of the tumor (5, 7, 10) significantly lessened the transmissibility of the Rous sarcoma by cells and by filtrates, and also that filtrability of the sarcoma was significantly higher in tumors induced by cells than in tumors induced by filtrates (10). On the other hand, the incidence, among birds, of metastases was not found to be significantly influenced by any of the above factors (10) but was significantly and directly related to the transmissibility of the tumors by filtrates and also by cells—phenomena which are, in turn, conditioned by the age factor.

The possibility that the effect of the age factor on cell transmissibility and formation of metastases is exerted through an effect on the virus has been discussed (10). In brief, it has been suggested that the virus might undergo changes in the aging *original* host which would finally result in its masking, as shown by nonfiltrability. This hypothesis would be considerably strengthened if it could be shown that a virus from an *original* tumor subjected to the effect of the age factor—but still retaining its filtrability—when injected into other *test* hosts induces tumors which behave in some respects like those from which the virus was obtained. The present investigation presents data pertinent to the point in the case of the formation of metastases.

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MATERIALS AND METHODS

Plymouth Rock chicks were used, as in a previous investigation (10). At the age of 14 days, these 479 birds were given subcutaneous injections of the Rous sarcoma materials as follows: 231 with 0.1 cc. of tumor cell suspension at a 1:5 dilution in each side of the breast and 248 with dilutions of tumor filtrates in several sites of the skin. We shall refer to these animals as "test chicks."

These chicks were given injections of materials from tumors of varying age grown in chickens of two age groups. The cell suspensions were derived from 41 adult chickens (15.9 ± 4.2 months) and 32 young ones (15.7 ± 5.9 days). The filtrates were obtained from seventeen adult chickens (15.9 ± 4.6 months) and 32 young ones (16.2 ± 6.6 days). We shall refer to these birds as "original hosts" and to their tumors as "original tumors."

All the test chicks developed at least one primary tumor. Appropriate analysis of the data showed that the number of primary tumors was not a factor in the development of metastases. The few cases where visceral growth was due to extension of the primary tumors were excluded. Almost all the test chicks died of their tumors. The time of survival after tumor inoculation was 23.7 ± 11.3 days for chicks injected with cell suspensions and 21.7 ± 5.6 days for chicks injected with filtrates. The incidence of grossly detectable metastases—and also of hemorrhagic lesions (9)—was carefully recorded at death. Histological examination was resorted to only in doubtful cases.

The statistical analysis of the results by one of us (P. M. F.) was carried out for the most part using Bliss's methods for the calculation of dose-response curves (1–4). In a previous study (10), these methods were applied to results of the same kind as those here considered.[1]

RESULTS

Of a total of 479 chicks injected with filtrates or cell suspensions, 170 (35.5 per cent) developed metastases. In Table 1 this incidence of metastases is shown in relation to characteristics of the

[1] The statistical analysis of the results can be found in detail in the thesis presented by Doctor P. M. Freire for the degree of Doctor of Public Health. The authors are very much indebted to Doctor C. I. Bliss for his most valuable suggestions in this respect.

original tumor material inoculated.[2] The possible influence of age of the original hosts, age of the original tumors, filtrability of the original tumors, and kind of material—cells or filtrates—inoculated into the test chicks were analyzed statistically.

The analysis of the data showed, as a main finding, that the number of test chicks with evident metastases decreased significantly with an increase in the age of the original tumors used for inoculation, this holding true even when only chicks inoculated with filtrates were considered. Furthermore, when the original tumors had grown in young chickens and the effect of different ages of these tumors was taken into account, test chicks with tumors induced by filtrates developed metastases at a significantly higher rate (45.6 per cent) than test chicks bearing tumors induced by cell suspensions of filtrable original tumors (26.4 per cent). This finding confirms an observation by Des Ligneris (6). On the other hand, no significant differences were associated with the age of the original hosts or with the filtrability of their tumors.

Next, we investigated the relation between the occurrence of metastases in the test chicks and the occurrence of the same lesions in the original hosts which provided the tumors inoculated into the test chicks. The results are shown in Table 2. There were fewer metastases in the test chicks when the inoculated tumor had not metastasized in the original host than when the original tumor had metastasized (29.3 per cent and 39.3 per cent, respectively). To evaluate the significance of this difference and the effect of age of the original tumors upon each group, dose-response curves were calculated as before.

This analysis showed that, when the original tumors had metastasized, an increase in the age of these tumors was significantly associated with a decrease in the incidence of metastases in the test chicks (from 52.9 to 23.3 per cent). With nonmetastasizing original tumors, the effect of the tumor age, although manifesting itself in the same general direction as in the former group, was not statistically significant (from 36.8 to 25.0 per cent). The difference between the incidence of

TABLE 1

INCIDENCE OF METASTASES IN TEST CHICKS DEVELOPING TUMORS

Age group of the original hosts	Age group of the original tumors (days)	Test chicks inoculated with cell suspensions: Filtrable original tumors — Tumor age in days, Geometric mean	Proportion of test chicks developing metastases	Per cent	Nonfiltrable original tumors — Tumor age in days, Geometric mean	Proportion of test chicks developing metastases	Per cent	Test chicks inoculated with filtrates — Tumor age in days, Geometric mean	Proportion of test chicks developing metastases	Per cent
Young	≤20	18.9	10/31	32.3				17.6	67/124	54.0
	21–40	29.2	10/32	31.3	21.0	1/8	12.5	29.2	9/31	29.0
	>40	50.0	4/28	14.3	52.8	3/9	33.3	50.0	7/27	25.9
	All ages	29.6	24/91	26.4	38.8	4/17	23.5	26.3	83/182	45.6
Adult	≤30	24.3	8/24	33.3	20.5	5/16	31.3	24.3	9/29	31.0
	31–40	33.3	7/17	41.2	33.8	5/21	23.8	33.3	9/22	40.9
	41–60	46.7	5/13	38.5	46.1	6/14	42.9	46.7	3/14	21.4
	>60	83.9	1/4	25.0	76.9	1/14	7.1	83.9	0/1	0
	All ages	34.0	21/58	36.2	37.5	17/65	26.2	34.0	21/66	31.8

TABLE 2

INCIDENCE OF METASTASES IN TEST CHICKS WITH TUMORS INDUCED BY FILTRATES OR CELL SUSPENSIONS

Age group of the original tumors (days)	Metastasizing original tumors — Age in days, Geometric mean	Proportion of test chicks developing metastases	Per cent	Nonmetastasizing original tumors — Age in days, Geometric mean	Proportion of test chicks developing metastases	Per cent
≤25	19.6	55/104	52.9	19.9	35/95	36.8
26–40	30.3	25/75	33.3	34.0	12/56	21.4
>40	49.0	14/60	23.3	56.5	16/64	25.0
All ages	29.4	94/239	39.3	34.8	63/215	29.3

[2] The organs showing metastases by order of frequency were: lungs, liver, kidneys, and heart, while occasional metastases were found in other locations. A number of the birds studied also showed hemorrhagic lesions (9). In this laboratory the incidence of both metastases and hemorrhagic lesions in a total of 1,871 injected at the age of 14–15 days with materials from the Rous sarcoma has been as follows: 34.6 per cent had only metastases; 22.4 per cent had only hemorrhagic lesions; and 43.0 per cent had both types of lesions combined.

metastases in test chicks inoculated with metastasizing original tumors and in those inoculated with nonmetastasizing ones was still apparent when the effect of the age of the original tumors was taken into consideration; however, this difference was not statistically significant.

DISCUSSION

When filtrates, as well as cells, from progressively aging *original* sarcomas were inoculated into *test* chicks, the incidence of metastases in these young hosts was significantly decreased as the age of the original tumor increased. It is clear, therefore, that the influence of the age factor on the metastasizing power of the test tumors took place through a change in the virus itself. Furthermore, this finding indicates the persistence of an effect of the age factor from the original host upon many generations of the virus even in the new host. The situation is the same as previously hypothesized for the virus in the process of masking (10).

It is curious why, with the methods followed, the effect of the age of the tumor on the formation of metastases was not evidenced in the original hosts. Possibly the metastases, presumably present in these hosts since the primary tumors were young, were not affected by the age factor, which would act in a cumulative way as the tumor ages (10). Yet, the injection of the virus from progressively aging original tumors into other hosts allowed isolation of the effect of tumor age from the original host.[3]

In agreement with the above interpretations, our experiments showed that the age of the tumors was of less consequence when these tumors did not metastasize in the original hosts. In such situations, the age factor is likely to have been significant since the initial stages of growth, thereby preventing early formation of metastases, and so further aging could add but little to the original effect. Likewise, although not statistically significant, the incidence of metastases in the test chicks was higher for metastasizing than for nonmetastasizing original tumors, which fact may indicate that the effect of the age factor was more pronounced in the case of tumors that did not metastasize in the original hosts.

[3] The restricting influence of tumor age on metastasis formation may seem in contradiction with the observations by workers (11, 12), including one of us (8), indicating that metastases are more commonly found in slowly growing tumors. The contradiction is only apparent: when the tumors grow fast, by the time the age factor begins to cumulate the animals die and a certain number have metastases; when the tumors grow slowly, this number of metastases will be found at the natural death of the animals plus a few more metastases that may or may not develop according to individual differences in the potency of the age factor.

The fact that significantly more metastases are observed in test chicks injected with filtrates than if injected with cells from an original tumor grown in young hosts (6) may indicate that, when freed from the aging cells of a previous host, the virus manifests a higher degree of vigor. The effect was not observed when the original tumors had grown in adult hosts in which the age factor presumably exerted a more intense and permanent influence on the virus.

The facts disclosed in this study supplement the discussion in the preceding paper (9) concerning the influence that qualitative changes in the virus, eventuating in masking, have on the properties of the malignant cell.

SUMMARY

The incidence of metastases from the Rous sarcoma in hosts inoculated in a given passage with filtrates and cells of the sarcoma was significantly decreased with an increase in the age of the tumors of the preceding generation from which the injected materials were obtained.

The finding is discussed in relation to the depressing action of the age factor on properties of the tumors through an effect on the causative virus.

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Growth and Regression of the Rous Sarcoma as a Function of the Age of the Host*

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Although there is extensive information concerning regression of the Rous sarcoma, the subject was restudied by us when it became clear that regression of the tumor was closely conditioned by the age factor—a factor which, as previously shown (11, 12), exerts such important effects on the tumor as masking its causative virus and suppressing its transmissibility by cells and its power to induce metastases. This paper reports the results of the study, together with other pertinent data.

Regression followed by varying degrees of immunity in several transplanted chicken tumors has been observed to occur spontaneously (2, 3, 5–7, 9, 16–19) or as a result of various treatments (4, 14, 15). No such regressions have been noted in young hosts, Gye and Purdy (13) observing only one case out of 6,000 young chickens bearing tumors. It has also been reported (1, 8, 14) that extirpation of a growing tumor is not followed by immunity.

MATERIALS AND METHODS

The present study is based on 192 adult chickens and 1,464 chicks of the Plymouth Rock breed. At the time of the first injection, with extracts or cells of the Rous sarcoma, the age of the chickens varied from 6 to 32 months, with a predominance of birds about 10 months old, while the age of the chicks was 15 days.

In this, as well as for the successive injections, the donor tumor was grown in chicks 10–20 days old. In every case the material inoculated was active, as shown by control injections. Saline was used for preparing the cell suspensions and the extracts, the latter being injected either unfiltered or after filtration through a Berkfeld N candle.

The amount of tumor material first inoculated and the sites injected varied widely. Thus, 102 adult chickens received 12–24 intradermal injections of 0.2-ml. of filtrates diluted at 1:40–1:40,000, while all the chicks received from four to six subcutaneous injections of 0.2 ml. of unfiltered extracts at 1:400. Of the rest of the adult chickens, 65 were injected with 1.5–4 ml. of filtrates at a 1:200 dilution, and 25 were injected with 1–2 ml. of cell suspension at 1:5, in the breast muscles.

In the case of adult chickens, some of the birds which did not develop tumors, or in which the tumors regressed after the first inoculation, were again challenged 1.5–7 months later, by the injection into the right breast muscles of 2–3 ml. of tumor cell suspension at a 1:5 dilution. The birds that resisted this second inoculation were again similarly challenged, and the procedure was repeated up to 8 times at intervals of from 3 weeks to 10 months when the experiment was discontinued.

In the case of chicks, some of the birds that did not develop tumors after the first inoculation were reinjected 35 days later—i.e., when the birds were 50 days old, with 1 ml. of unfiltered tumor extract at a 1:20 dilution into each side of the breast. The few birds that did not develop tumors were given a third injection at 1 ml. of cell suspension at 1:5 in each side of the breast, 33 days after the second inoculation—i.e., when the birds were 83 days old. The only survivor was injected a fourth time, 45 days later, therefore, at the age of 4 months, as done in the third injection.

RESULTS

Of the 192 adult chickens first inoculated, 27 (14 per cent) did not respond to the tumor injection, and eighteen of them served for the successive reinoculations. The remaining 165 chickens developed one or several tumors. Of these, the tumors in 140 (87 per cent) grew progressively until the death of the host, while in the remaining 25 birds (13 per cent) the tumors underwent regression. Eighteen of the birds were used for reinoculation. It is to be noted that, since 98 of the chickens were killed 3–12 weeks after inoculation, some of the tumors in these hosts might also have regressed in the course of time.

Of the 1,464 chicks first inoculated, 136 (9 per cent) did not respond to the tumor, and 48 of them served for successive reinoculations. The remaining 1,328 birds (91 per cent) developed one or several progressively growing tumors without any regressions being observed.

It is seen that the percentage of adult chickens not responding to the first inoculation was higher than that of young chickens, but the statistical significance of the difference is doubtful (χ^2 =

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3.836; P = 0.05). Also, the incidence of tumors was higher when cell suspensions were injected as compared to filtrates, but here again the differences were not significant ($\chi^2 = 2.595$; P = 0.27).[1]

Another point is that there were no significant differences in the incidence of regressions, depending on the route of inoculation—intradermal or intramuscular ($\chi^2 = 0.284$; P = 0.85). This may be at variance with results by other workers (5), but the experimental conditions were not identical in the two studies.

The results of reinoculating both chicks and chickens are given in Chart 1 in which the symbols representing the reactions of each individual bird throughout the successive inoculations are arranged vertically along straight lines, with each bird keeping its position in the horizontal group.

From data in the chart, the following is clear: Irrespective of their age, the birds which did not respond to the first tumor inoculation did not show any increased resistance to the second inoculation, since tumors developed in 89 per cent and 85 per cent in adult and young hosts, respectively; and, past the fourth inoculation, only one adult chicken proved to be immune to the tumor.

On the contrary, of the birds in which tumors regressed after the first inoculation—and this event took place exclusively in adult hosts—only 39 per cent and 7.6 per cent responded to the second and third inoculations, respectively. This is in contrast to the 96 per cent of responses to the first inoculation. The eleven remaining birds (a twelfth bird died accidentally) proved to be immune to all the successive reinoculations. In adult chickens, after the second inoculation, tumor regression occurred, too, but at a significantly higher ratio in the birds that had regressing tumors (28 per cent) than in those not responding to the tumor (12 per cent) after the first inoculation ($\chi^2 = 7.706$; P = 0.005).

In young chickens, in contrast to what was observed after the first inoculation when the hosts were 15 days old, tumor regression occurred in two cases (4.8 per cent) following the second inoculation, but by that time the hosts were already 50 days old. Accidental death prevented further testing of the resistance of the two birds to the tumor,

In summary, a solid immunity against the tumor developed only in aging hosts in which the tumor regressed after an initial growth. Irresponsiveness to the tumor first inoculated was largely independent of the age factor and in no way implied permanent resistance to the tumor.

[1] It was reported elsewhere (10) that young hosts had a greater susceptibility than older ones to the Rous sarcoma virus. However, the differences were clearly noticeable only when the same amounts of high dilutions of the same tumor extract were injected intradermally at the same time into young and adult hosts.

Some Additional Observations

The tumors regressing after the first inoculation ranged in size from a few square millimeters to 60 square centimeters. Apparently, the mass of reabsorbed tumor tissue did not determine the degree of resulting immunity, for we had examples of solid immunity following regression of the smallest tumors and of tumor development on further inoculation, following regression of some of the largest tumors.

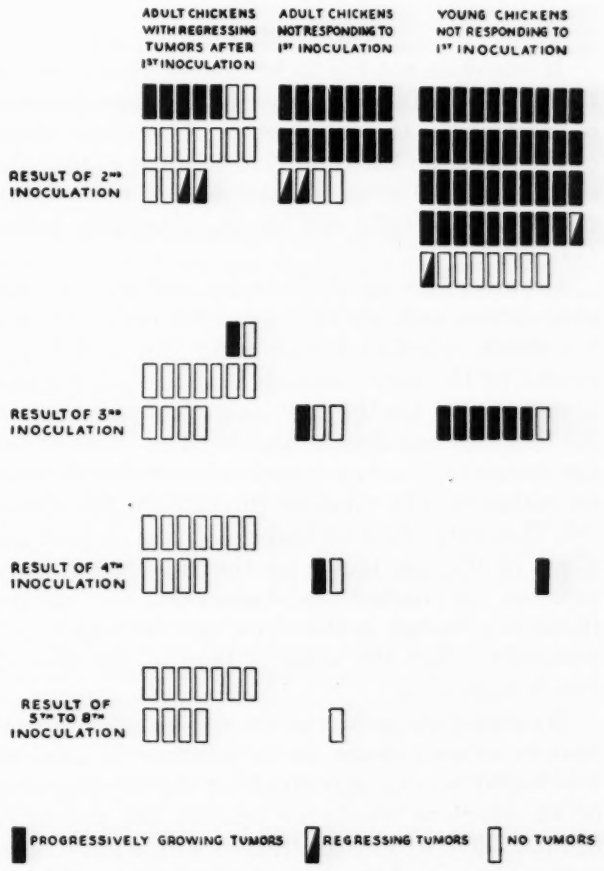
ADULT CHICKENS WITH REGRESSING TUMORS AFTER 1ST INOCULATION
ADULT CHICKENS NOT RESPONDING TO 1ST INOCULATION
YOUNG CHICKENS NOT RESPONDING TO 1ST INOCULATION
RESULT OF 2ND INOCULATION
RESULT OF 3RD INOCULATION
RESULT OF 4TH INOCULATION
RESULT OF 5TH TO 8TH INOCULATION
PROGRESSIVELY GROWING TUMORS
REGRESSING TUMORS
NO TUMORS

CHART 1.—The reactions in individual chickens to successive inoculations of the Rous sarcoma.

The inoculations of tumor cells into the immune chickens brought about sharp reactions which subsided after several days. Histological study showed the ordinary signs of inflammation, with conspicuous infiltration by lymphoid-like cells, and pronounced muscle necrosis.

The eleven chickens that resisted at least four inoculations of tumor died or were killed at ages varying from $1\frac{1}{2}$ to $4\frac{1}{2}$ years. Eight of them bore no tumors or lesions worth mentioning. Two of the birds that died had tumors in the ovary which had

induced metastases either in the liver or the intestine; these tumors proved to be tubular adenomas. The last chicken had in the left breast a large, firm not viscid tumor, with extensive necrosis. This growth proved to be a fibrosarcoma different from the Rous sarcoma. However, in view of the location of the lesion one can presume that this tumor was a late and much changed growth resulting from the fourth and last inoculation of tumor cells.

DISCUSSION

If one does not try to be precise about the nature of the age factor, one can regard the regression of sarcomas as the culmination of previous effects of the factor on the tumor, which lead to masking of its causative virus and suppression of its transmissibility by cells and its metastasizing power (11, 12).

However, the age factor may well be of a complex nature, and, with the available data, one cannot decide whether tumor regression is or is not caused by the same component of the factor which is responsible for the previous effects on tumors. Neither can one decide whether the effect of the age factor on tumor regression is exerted through an action on the virus or directly on the tumor cell. However, since we know (12) that at least one effect of the age factor on the tumor, e.g., suppression of metastasis formation, is exerted through a change in the virus, one can admit as a possibility that the same is true in the case of tumor regression.

Whatever the nature of the age factor, it is clear that in avian tumors, as in infection in general, this factor is very important for the development of an effective resistance against the causative agents. This is to be contrasted with a state of irresponsiveness of the hosts to the tumor, which is largely independent of the age factor. Such state—the cause of which is entirely unknown—does not imply at all that the hosts are either resistant or more apt to develop resistance to the sarcoma.

Finally, the fact that epithelial malignancy developed in birds immune to the Rous sarcoma implies the existence of important antigenic differences between these two types of tumors.

SUMMARY

Regression of actively growing Rous sarcomas occurred in at least 15 per cent of 192 adult or old chickens, whereas it never occurred in 1,464 chicks 15 days old when injected.

Tumor regression was generally followed by resistance manifested either by lack of growth, or by a still higher rate of regressions following further tumor inoculations. However, two of eleven birds immune to the sarcoma developed malignant epithelial tumors of the ovary.

Irrespective of their age—in 9 per cent of chicks and 13 per cent of adult chickens—a number of hosts failed to respond to a first tumor inoculation but responded to reinoculation just as normal chickens do. This state of nonresponsiveness, therefore, is different from the efficient immune reactions linked with the age factor.

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Growth of Human Tumors in Cortisone-treated Laboratory Animals: The Possibility of Obtaining Permanently Transplantable Human Tumors*†

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A preliminary paper reported that human neoplasms, implanted as either a suspension of cells or a fine mince, often proliferated extensively for 8–10 days in the subcutaneous tissues of adult, x-radiated heterologous hosts before changes associated with regression occurred; they did not survive in nonirradiated animals (2). Subsequently, it was noted that human tissues, similarly implanted in x-radiated rats of weanling age, could grow progressively for 12–14 days or longer (3, 4), a period which allowed the more vigorously growing tumors to be of practical value for the testing of chemotherapeutic agents (6). More recently, it was learned that human cancer cells showed a higher percentage of "takes" and proliferated considerably longer in x-radiated weanling rats treated with cortisone than in hosts which had received roentgen treatment alone (5). The present paper is concerned with observations on the growth and serial transplantation of human tumors in both x-radiated and nonirradiated animals (rats and hamsters) treated with cortisone. The findings have been favorable enough so that the ultimate goal of obtaining one or more human neoplasms that can be maintained as serially transplantable tumors in large numbers of laboratory animals, seems in sight.

MATERIALS AND METHODS

Female albino rats (CF Wistar, Carworth Farms) and hamsters (*Mesocricetus auratus*, Lakeview Hamster Colony) of weanling age and weighing approximately 45 gm. were used for these experiments. The animals were of one sex for the purpose of uniformity and for convenience in caging. X-radiated rats were given a total of 300 r in two doses of 150 r each on consecutive days, while the x-radiated hamsters usually received one dose of 200 r. Two 180-kvP tubes (one above, one below the animals) were employed to save irradiation time, though one tube (above) was just as satisfactory. The animals received 25 ma. from each of the two tubes filtered with ½ mm.Cu. and 1.65 Al filters (not parabolic) at a TSD of 40 cm. (73 units per minute). Animals were implanted with tumors in the 1–5-day period following the last treatment with radiation.

Tumors were obtained as fresh and sterile as possible from the operating rooms (autopsy material was not used) and were minced finely enough with scalpels so that they could be implanted by injection through a No. 16 or No. 18 needle. Ringer's solution, buffered to a pH of approximately 7.3 and with 6 mg of glucose and 200 units of penicillin added/cc, was used as suspending medium. All initial implantations were made in the subcutaneous tissues (flanks) of rats, as it had been found previously that these hosts were more uniform in their reaction to the implantation of any given human tumor than were hamsters (3). Transfers of tumor into rats were by implantation of a mince obtained from material of the previous generation whether grown in rats or hamsters. (Prior experiments had also indicated that the subcutaneous implantation of a tumor mince into the rats gave more favorable results than did trocar pieces or sieved cell suspensions). Transfers into hamsters, which had been anesthetized with nembutal, were by trocar pieces implanted in the cheek pouches, according to the method of Lutz *et al.* (1). Each group of animals reported as positive or negative for tumor growth consisted of at least two animals and usually three or more.

Rats treated with cortisone were given four subcutaneous injections of the drug as distant as possible from the tumor: 3 mg/dose for the x-radiated, 6 mg. for the nonirradiated animals. The initial dose was on the day of, and immediately after, tumor implantation, with subsequent injections on alternate days following. The 3-mg. dose in the x-radiated hosts produced results comparable to those obtained with the 6-mg. dose in nonirradiated hosts. Daily injections or increases in the doses cited often proved to be toxic to the animals, and less beneficial to the growth of the tumors which failed to become stromatized. Hamsters were more sensitive to cortisone than the rats, and an optimal dosage has not been established as yet. For the work reported in Charts 1 and 2, the hamsters were treated with one injection of cortisone given on the day of implantation (3 mg. if x-radiated, 6 mg. if nonirradiated) and a second similar injection 1 week later.

The term "normal" has been used to designate a nonirradiated animal.

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† The cortisone acetate (Cortone, Merck) used for these experiments has been supplied through the courtesy of Merck & Co., Inc., Rahway, N.J.

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RESULTS AND DISCUSSION

One hundred and one human tumors were implanted in weanling rats. With the exception of melanomas and mammary adenocarcinomas, which will be reported as a group in another paper, they were consecutively obtained. The first 27 neoplasms that were received were implanted in x-radiated rats only; these rats were killed on the 10th–13th days after implantation (Table 1, Group 1). Slightly over 50 per cent (fourteen) of the 27 tumors grew in the x-radiated control animals, whereas almost 90 per cent (24) were positive in similarly x-radiated animals treated with cortisone (Figs. 1–3). The epidermoid carcinomas grew in 56 per cent of the x-radiated control rats; this was comparable to our previous report that almost 60 per cent of this type tumor had a favorable growth response in x-radiated weanling rats (3). The next eighteen neoplasms were implanted in both x-radiated and normal rats. Half the animals in each group were treated with cortisone (Table 1, Group 2). Again, a greater number of tumors grew in the x-radiated hosts treated with cortisone than in the x-radiated controls: seventeen out of eighteen, as compared to ten out of eighteen. Of even more interest, however, was the finding that seventeen of the eighteen tumors implanted in nonirradiated hosts receiving cortisone also grew (Fig. 4), while none survived in the normal control rats. Of the total of 45 tumors implanted in x-radiated hosts (Table 1, Group 1 plus Group 2) 50 per cent grew in the control x-radiated hosts, as compared to 90 per cent in the cortisone-treated x-radiated hosts, since 24 tumors were positive in the former and 41 in the latter.

It was apparent from microscopical examination that the tumors in the cortisone-treated hosts were uniformly larger and more vigorous than those in the animals x-radiated only. It thus seemed likely that they would survive longer than

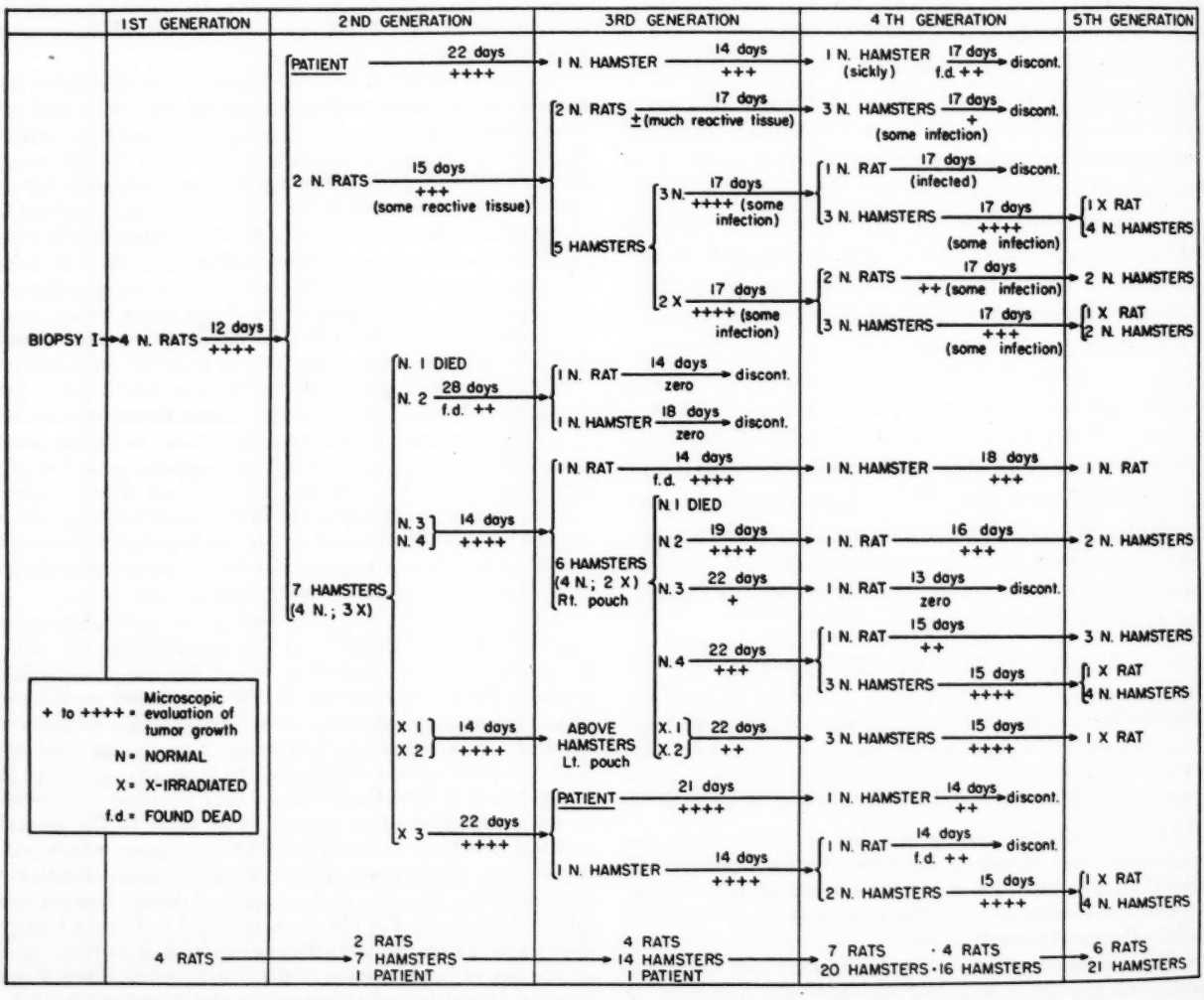

CHART 1.—Transfer record of a human epidermoid carcinoma grown in nonirradiated ("normal") and x-radiated rats and hamsters treated with cortisone. The original material was a biopsy specimen from an inguinal node containing metastatic tumor; primary tumor was in the cervix. Some necrosis in biopsy.

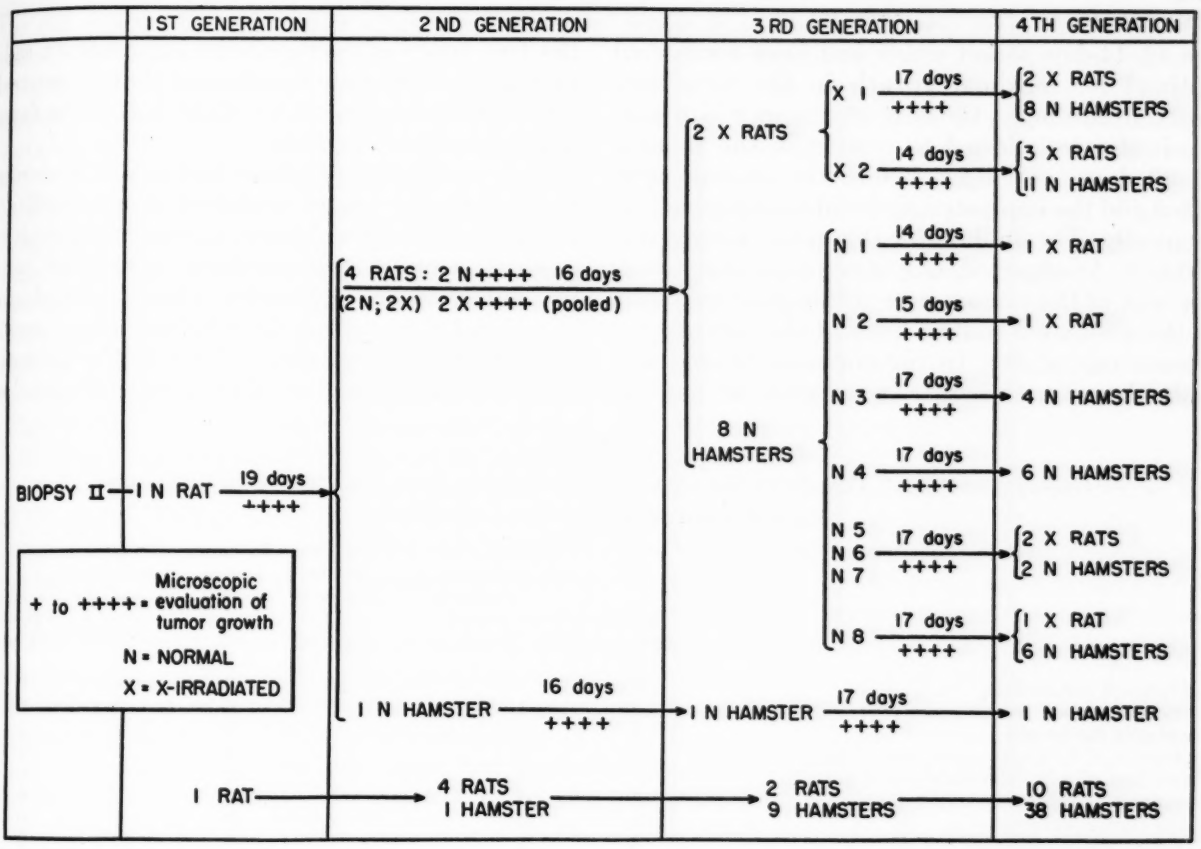

CHART 2.—As Chart 1. Second biopsy specimen, same patient, also from an inguinal node. No necrosis in biopsy

TABLE 1

GROWTH OF HUMAN TUMORS IN X-RADIATED AND NORMAL RATS TREATED WITH CORTISONE

Animals killed 10–13 days after implantation

	No. implanted	No. positive in x-radiated control rats	No. positive in x-radiated plus cortisone	No. positive in normal control rats	No. positive in normal rats plus cortisone	No. failed to grow in any rats
Group I (Implanted in x-radiated rats only):						
Epidermoid carcinomas	18	10	16			2
Oat-cell carcinoma of lung	1	1	1			0
Giant- and spindle-cell carcinoma of thyroid	1	1	1			1
Adeno-cystic carcinoma of parotid	1	0	1			0
Mixed tumor of parotid	1	1	1			0
Adenocarcinoma of rectum	2	0	1			0
Adenocarcinoma of pancreas	1	1	1			0
Synovioma	1	0	1			0
Reticulum-cell carcinoma	1	0	1			0
Totals (Group I)	27	14	24			3
Group II (Implanted in both x-radiated and normal rats):						
Epidermoid carcinomas	13	7	12	0	12	1
Carcinoma of ovary	1	1	1	0	1	0
Papillary and follicular carcinoma of thyroid	1	0	1	0	1	0
Warthins tumor of parotid	1	1	1	0	1	0
Adenocarcinoma of caecum	1	0	1	0	1	0
Spindle-cell carcinoma	1	1	1	0	1	0
Totals (Group II)	18	10	17	0	17	1
TOTALS (Groups I and II)	45	24	41	0	17	4

the 12–14-day period which had been considered optimal for maximal growth in the x-radiated hosts. Accordingly, the next 56 tumors which were received were allowed to remain in the rats for 14–20 days, or longer, before the animals were killed and the implanted material examined microscopically. The findings for this group are given in Table 2. As expected, only a comparatively small per cent of the tumors were still in good condition in the x-radiated control hosts at this later period (eleven out of 56). In the cortisone-treated animals, however, whether x-radiated or not, 49 (88 per cent) were growing progressively. No tumor cells were seen in normal control hosts.

TABLE 2

GROWTH OF HUMAN TUMORS IN X-RADIATED AND NORMAL RATS TREATED WITH CORTISONE

Animals killed 14–20 days after implantation

	No. implanted	No. positive in x-radiated control rats	No. positive in x-radiated plus cortisone	No. positive in normal control rats	No. positive in normal rats plus cortisone	No. failed to grow in any rats
Epidermoid carcinomas	39	6	36	0	36	3
Adenosquamous carcinoma of cervix	1	1	1	0	1	0
Anaplastic carcinoma	1	0 (28 days)	1	0	1	0
Papillary and follicular carcinoma of thyroid	1	0	1	0	1	0
Giant- and spindle-cell carcinoma of thyroid	1	0	1	0	1	0
Carcinoma of kidney	1	0	1	0	1	0
Wilms tumor (kidney)	1	0	1	0	1	0
Warthins tumor (parotid)	1	1	1	0	1	0
Thyroid adenoma	1	0	1	0	1	0
Papillary adenocarcinoma of ovary	1	1	1	0	1	0
Adenocarcinoma of rectum	2	1	1	0	1	1
Thymoma	1	0	1	0	1	0
Hemangiopericytoma	1	1	1	0	1	0
Rhabdomyosarcoma	1	0	1	0	1	0
Hypernephroma	1	0	0	0	0	1
Fibrosarcoma (patient aged 91)	1	0	0	0	0	1
Reticulum-cell sarcoma	1	0	0	0	0	1
Totals	56	11	49	0	49	7

In total, approximately 90 per cent (90) of the 101 human tumors implanted in cortisone-treated x-radiated hosts and 89 per cent (66) of the 74 tumors implanted in cortisone-treated nonirradiated hosts were growing 12–20 days after implantation of the tumors.

Six of the epidermoid carcinomas had been implanted in enough rats so that some animals were available for examination at a still later period. It was found that, if an additional injection of cortisone was given to the rat host 10–14 days after the final injection in the initial series of four doses, the tumor borne by this animal not only stayed alive but was still growing 28–30 days after implantation. Regressive changes or death of the tumor cells occurred where this supplementary injection was not given.

A comparison of the extent of tumor growth in the two types of cortisone-treated hosts (x-radiated and nonirradiated) indicated that, in general, they were the same, with a slight balance in favor of the x-radiated animals.

As a result of the findings just noted, it seemed likely that the use of cortisone would facilitate serial transfer of the human tumors in laboratory animals. One of the problems associated with previous work on the transfer of human neoplasms in x-radiated hosts was the relatively short period that the tumors remained viable in the animals. Since a certain number of cells were always lost with each transfer, it was necessary for the tumor to multiply sufficiently in any one generation to make up for this deficiency. This did not often occur. A further difficulty encountered was the hyalinization of old stromal material which, in x-radiated rats, was transferred from generation to generation along with the tumor cells. Instead of dying, to be replaced with the new host stroma, as is the usual case in transplantable tumors, it survived in poor condition from one x-radiated rat to another and eventually, in the third or fourth generation, tended to choke off the tumor cells and allow no penetration of new supporting tissue or blood vessels. It was hoped that use of cortisone would enhance and prolong the growth of the tumors so that the number of cells lost during transfer would not determine the fate of the transplant. Furthermore, it was thought that cross transfers between animals of two species, such as rats and hamsters, might eliminate retention of

the old stroma and therefore allow new stromal tissue to support the tumor cells.

It is not at all certain that this reasoning is correct. However, it was found that if the more vigorous tumors were selected, serial transplants were accomplished with comparative ease in cortisone-treated hosts. One adenocarcinoma (Figs. 5–8) has been transferred for four generations at the present writing, and an epidermoid carcinoma, originally obtained on two separate occasions as small biopsy specimens, each approximately 4 × 4 × 5 mm., now gives promise of becoming a permanently transplantable tumor (Figs. 9–11). It has increased in amount, so that in the fourth generation ten rats and 38 hamsters are carrying excellent tumors, all derived from one of the small biopsy specimens, minced and implanted in a single cortisone-treated rat. Details of the transfer records of the two biopsies are given in Charts 1 and 2.

The first biopsy (Fig. 12) contained some necrosis and, possibly, a low grade infection which has appeared in some of the animals of the later generations. In spite of the discard of certain animals because of this infection, there has been a notable consistent increase in material from the first (Figs. 13, 14) to the fifth generations (Fig. 16). In order to determine whether the tumor had maintained its human characteristics in spite of its sojourn in animals (a matter which is of the utmost importance in determining the future value of transplantable human tumors carried in the laboratory), two back-transfers of this tumor (trocar-size pieces) to the original donor patient were made. One was done in the second generation, another in the third (Chart 1). When removed, approximately 3 weeks after their subcutaneous insertion, they were in excellent condition, with every evidence of acclimatization (Fig. 15). Naturally, such an experiment is strictly limited and was only permissible in this instance because of the completely inoperable and terminal condition of the patient.

The second biopsy from the same patient (Chart 2) was composed of solid tumor with no evidence of necrosis or infection. It has proliferated rapidly and transferred very well. Large solid tumors, free from necrosis, have been produced (Figs. 9–11) from every transplant. At the present time, it is being carried in x-radiated rats and nonirradiated hamsters, all treated with cortisone. Normal, untreated animals, which, in our experience, have never supported proliferative and progressive tumor growth, have not as yet received implants. This procedure has been followed in order that no tumor might be lost and that a uniform base-line might be established for an increase in material from one generation to another in the cortisone-treated hosts. We have now obtained enough tumor so that untreated animals can also be used as hosts. It will be desirable, of course, to establish a tumor in such animals, if possible.

It should be noted that even though 90 per cent of human tumors implanted in the cortisone-treated hosts survived and proliferated, only a comparatively small per cent appear vigorous enough for permanent cultivation (about one of every fifteen to twenty tumors implanted in our series). This is not too unfavorable when compared to the transfer record of spontaneous tumors in laboratory animals.

The importance of the cross transfers between rats and hamsters in promoting tumor growth has not as yet been determined. There appears to be some tendency in the rat-to-rat transfers for the presence of an increasing proportion of stroma to tumor—some of the stroma being apparently retained from the previous generations. This is not true if cross transfers are made, and at the present writing does not appear to occur in the hamster-to-hamster transfers. In a number of tests (not tabulated), the cortisone-treated rats were more susceptible hosts to the original human tumors than were the hamsters. After the first generation the cortisone-treated hamsters supported the tumor growth as well as did the rat hosts. In none of the tumors which grew was there much evidence of host reactive tissue (Fig. 4), unless they were carried longer than the periods cited. Vascularization was excellent (Fig. 14), and mitosis was most extensive and always greater than in the original specimen (Fig. 7).

Four tumors were implanted in adrenalectomized x-radiated and nonirradiated rats, as well as in the usual hosts. Growth in this series occurred in all the cortisone-treated animals, whether adrenalectomized or not, and in none of the other rats. Occasionally, some slight cortisone-like effect has been obtained with daily injections of ACTH, but, in general, this substance appears to promote reactive tissue. Certainly this last study is not complete, for the amount of drug to be given, as well as the problems involved in the inter-relationship of ACTH with cortisone, desoxycorticosterone, and other adrenal steroids, remains as yet undetermined. There is also no certainty that the doses of cortisone employed in the experiments of this paper are optimal for establishing and maintaining human tumor growth in the heterologous hosts. Considerably more work is being planned on this problem. The value of pretreatment of animals with the cortisone, as well as the mixing of

cortisone and tumor mince at the time of implantation, are being investigated.

SUMMARY

Approximately 90 per cent of 101 human tumors implanted in cortisone-treated x-radiated rats survived and proliferated for 12–20 days. In some instances they were maintained as long as 30 days. A similar percentage of tumors implanted in cortisone-treated nonirradiated rats were also positive for growth. None survived in normal control animals.

One biopsy specimen of a human epidermoid carcinoma, implanted originally in a single cortisone-treated rat, has so increased in the fourth transfer generation that tumors are being carried by ten rats and 38 hamsters, all cortisone-treated. It is hoped that this neoplasm can be maintained permanently as a transplantable tumor in laboratory animals.

ACKNOWLEDGMENTS

The author wishes to express her special gratitude to Miss Alice Gale and Miss Joan Livoti for their technical assistance and to Dr. Chester Southam for his valuable aid in arranging for the biopsy material and back-transplants noted in Charts 1 and 2.

ADDENDUM

Since this paper was written, the small biopsy specimen of Chart 2, originally implanted in one rat, is in its eighth and ninth transfer generations. It is now being carried by 150 laboratory animals (rats, hamsters, and mice).

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6. Toolan, H. W., and Moore, A. E. Oncolytic Effect of Egypt Virus on a Human Epidermoid Carcinoma Grown in X-Irradiated Rats. Proc. Soc. Exper. Biol. & Med., **79**:697–702, 1952.

Fig. 1.—Human epidermoid carcinoma (original specimen from lung), 12 days after implantation as a fine mince in the subcutaneous tissues (flank) of an x-radiated rat treated with cortisone. H and E (Hematoxylin and Eosin) stain. ×160.

Fig. 2.—Higher magnification of Figure 1. The numerous mitoses are indicated by arrows. H and E stain. ×450.

Fig. 3.—Human metastatic squamous-cell carcinoma (primary in floor of mouth), 12 days after implantation in the flank of an x-radiated rat treated with cortisone. Arrow points to blood vessel. H and E stain. ×140.

Fig. 4.—Human metastatic ovarian carcinoma, 11 days after implantation in the flank of a nonirradiated rat treated with cortisone. Note the lack of reactive cells in the rat tissue (arrows) about the tumor. H and E stain. ×160.

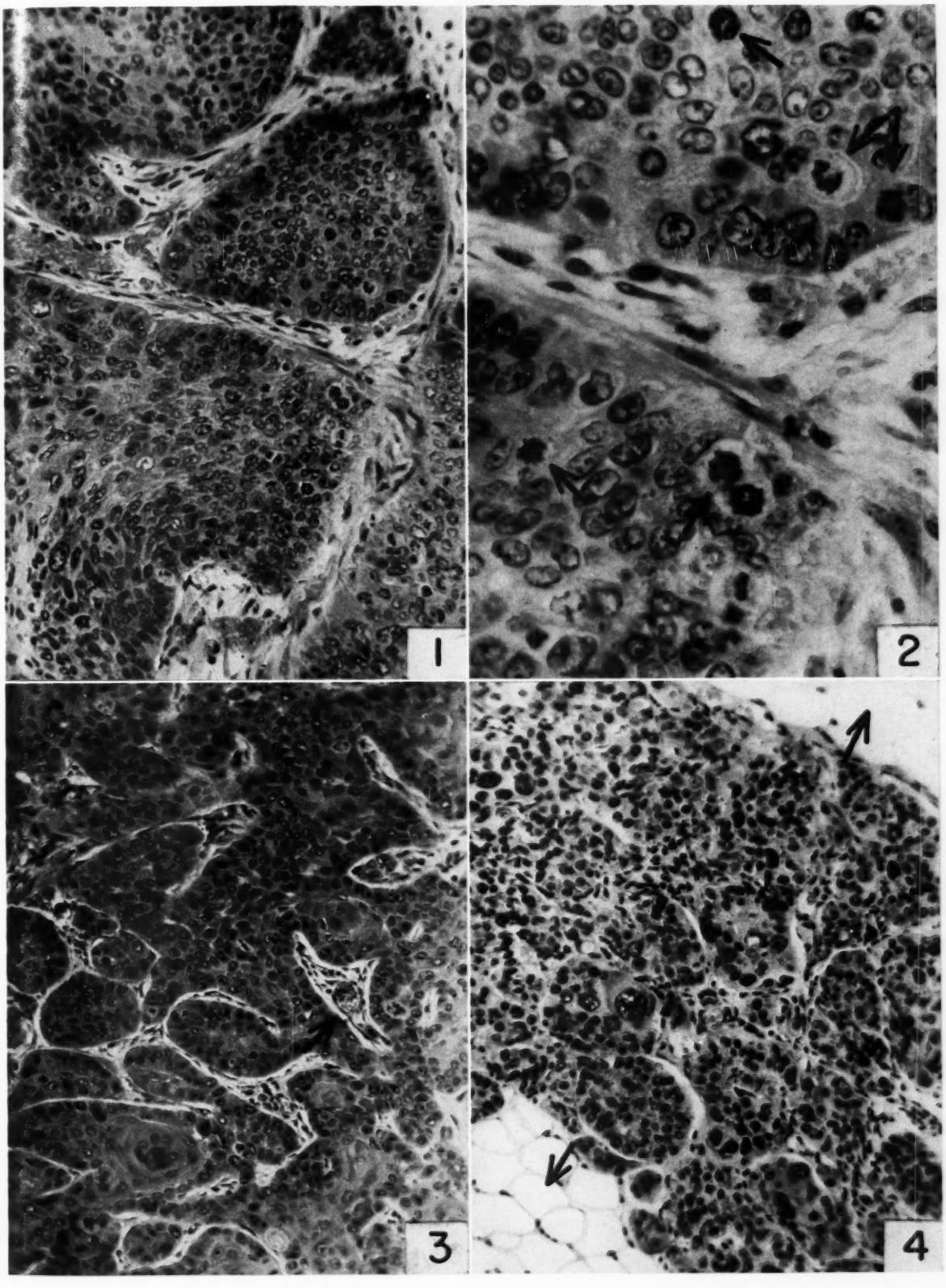
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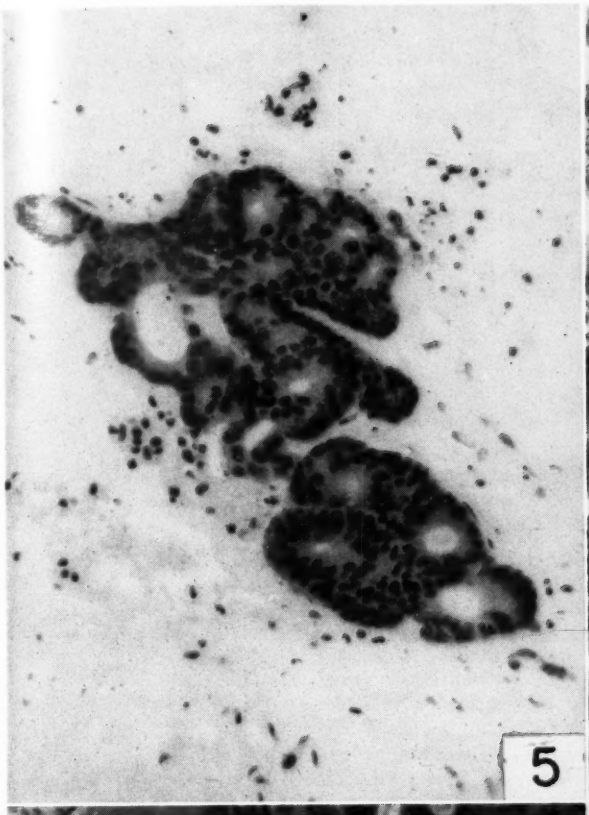
FIG. 5.—Original human tumor: metastatic adenocarcinoma in a small nodule removed at operation from the liver of a 60-year-old female (primary, unknown; probably in large bowel). There were comparatively few of these tumor foci per c. cm. of the specimen. H and E stain. ×140.

FIG. 6.—Tumor from Figure 5, 12 days after implantation in the subcutaneous tissues of a nonirradiated rat treated with cortisone. The tumor is now very compact with very little stroma. H and E stain. ×160.

FIG. 7.—As Figure 6. Another view at higher magnification. Arrows point to numerous mitoses. H and E stain. ×360.

FIG. 8.—Tumor of Figures 5–7 at the end of four generations (60 days) in nonirradiated animals treated with cortisone. First two generations in the subcutaneous tissues of rats; second two generations in the cheek pouches of hamsters. H and E stain. ×160.



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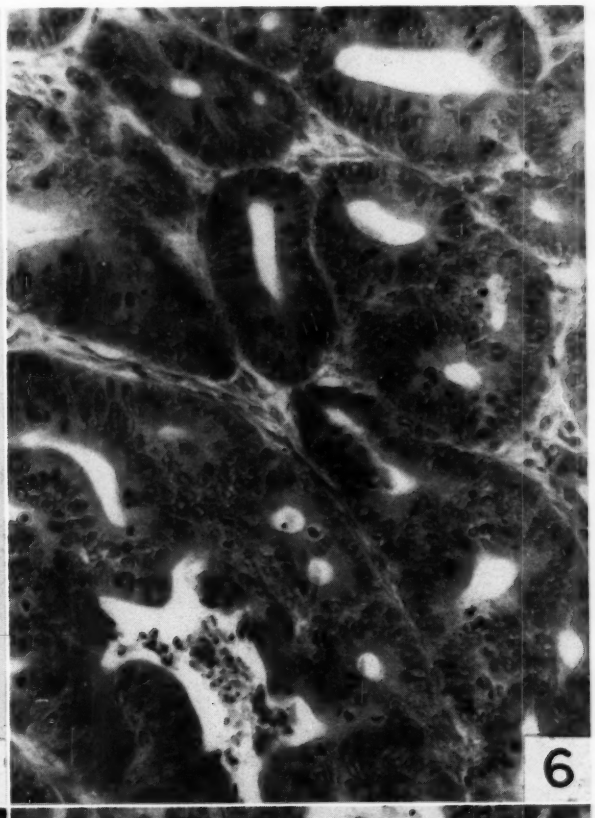

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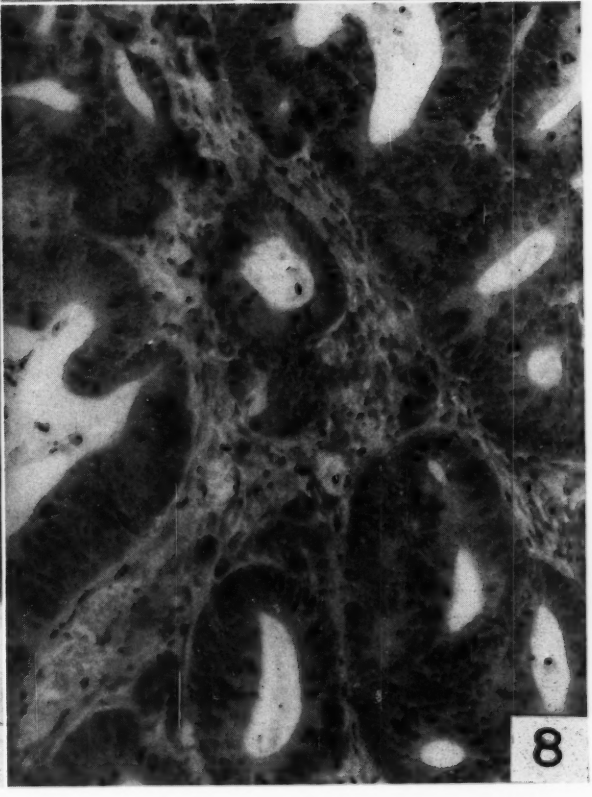

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FIG. 9.—Third generation of human epidermoid carcinoma grown in the subcutaneous tissues of weanling rats. (Chart 2, x-radiated rat No. 1, text.) For the first two generations the tumor was grown in nonirradiated rats and during the third, in an x-radiated rat; all were treated with cortisone. This generation was 17 days old; the total number of days since removal from the patient was 52.

FIG. 10.—As Figure 9, third generation, x-radiated rat No. 2 (Chart 2, text). The tumor has been pried away somewhat from the rat supporting tissue for photographic purposes.

FIG. 11.—As Figure 9, third generation, cheek pouch of cortisone-treated hamster No. 8 (Chart 2, text). First two generations in cortisone-treated rats.

FIG. 12.—Original biopsy specimen of the human epidermoid carcinoma used for transplants of Chart 1 (text). The biopsy was obtained from metastatic tumor in an inguinal node. Primary tumor was in cervix. H and E stain. ×140.

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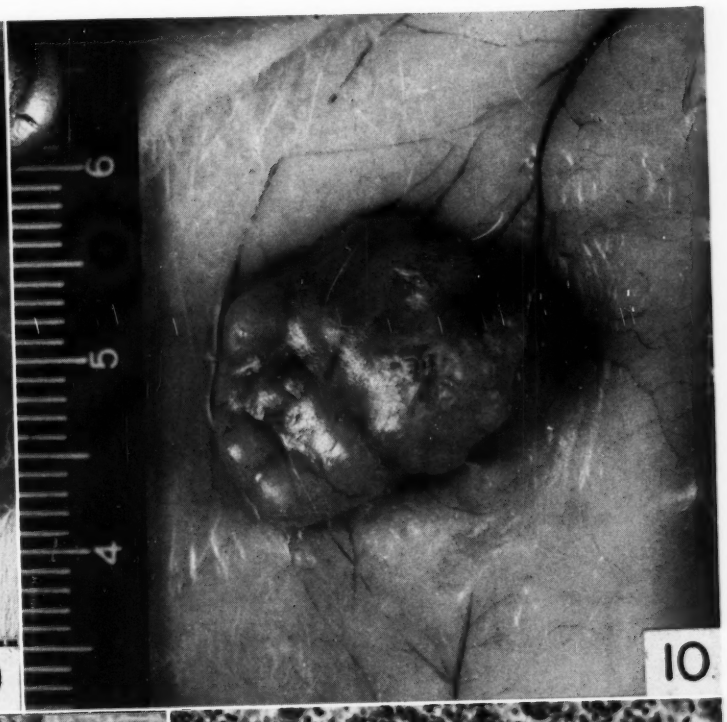

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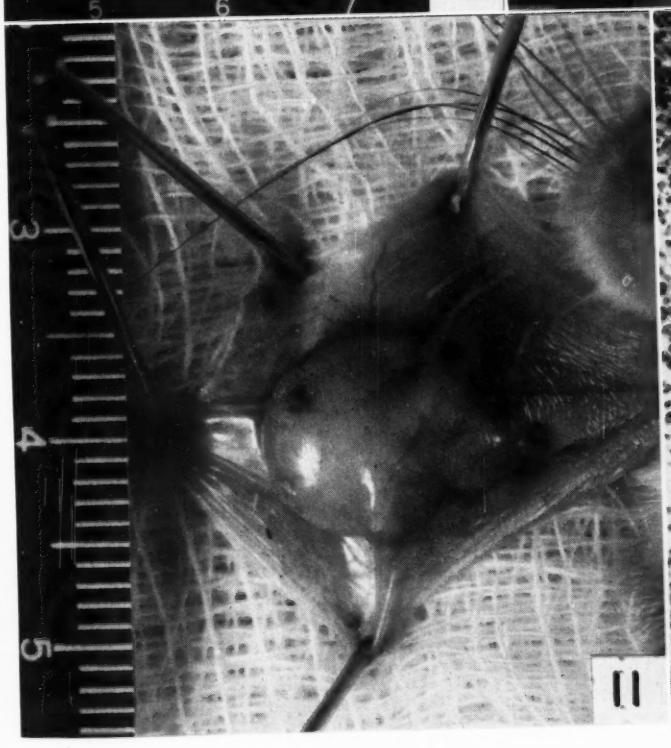

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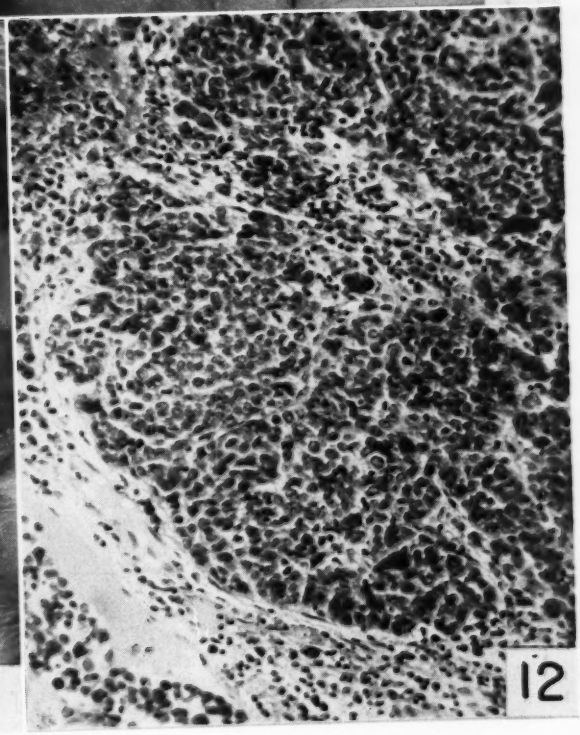

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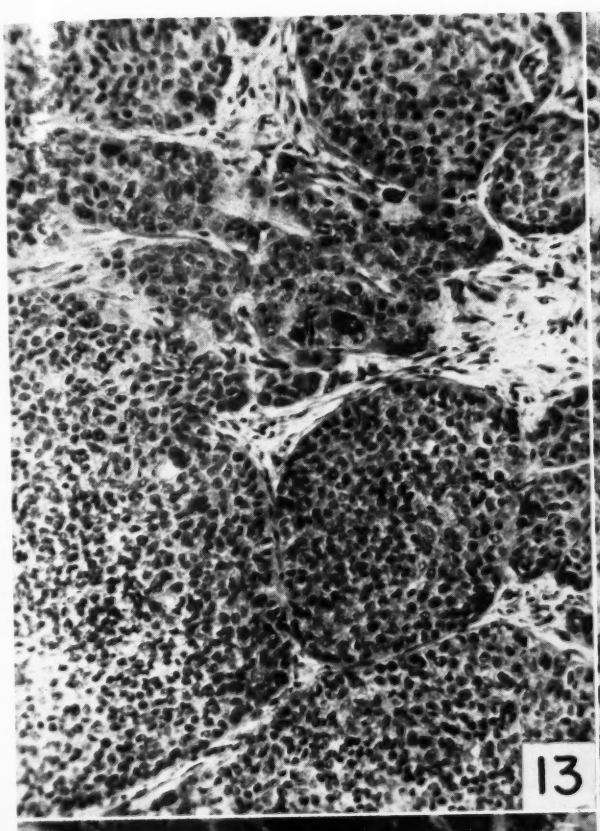
FIG. 13.—The human epidermoid carcinoma of Figure 12, 12 days after implantation in a nonirradiated rat treated with-cortisone. (Chart 1, text.) H and E stain. ×140.

FIG. 14.—Higher magnification of Figure 13 showing good vascularization (arrow). H and E stain. ×400.

FIG. 15.—Tumor of Figure 12 at the end of the third generation which was a back-transfer (21 days duration) to the patient (Chart 1, text). The first generation (12 days) was in the rat of Figures 13 and 14, the second generation (22 days) in the cheek pouch of a cortisone-treated hamster. The tumor does not appear to have been altered in relation to the patient by its sojourn in the laboratory animals. H and E stain. ×90.

FIG. 16.—Tumor of Figure 12 at end of the fourth generation in cortisone-treated animals. The first two generations were in rats; the second two in hamsters. Total number of days since removal from patient was 61. H and E stain. ×140.



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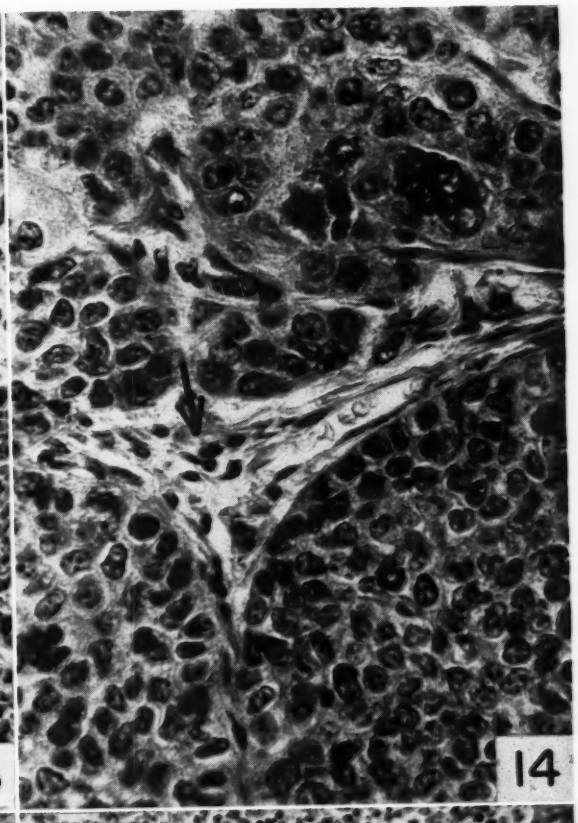

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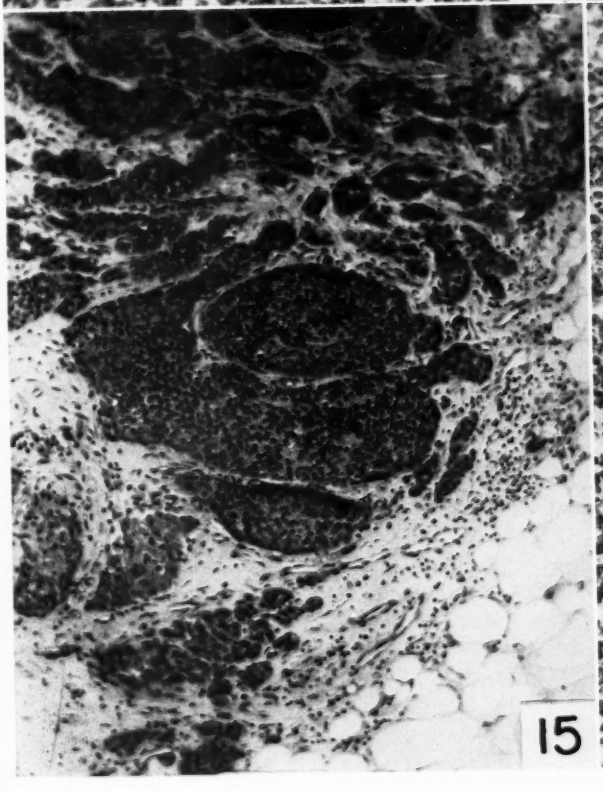

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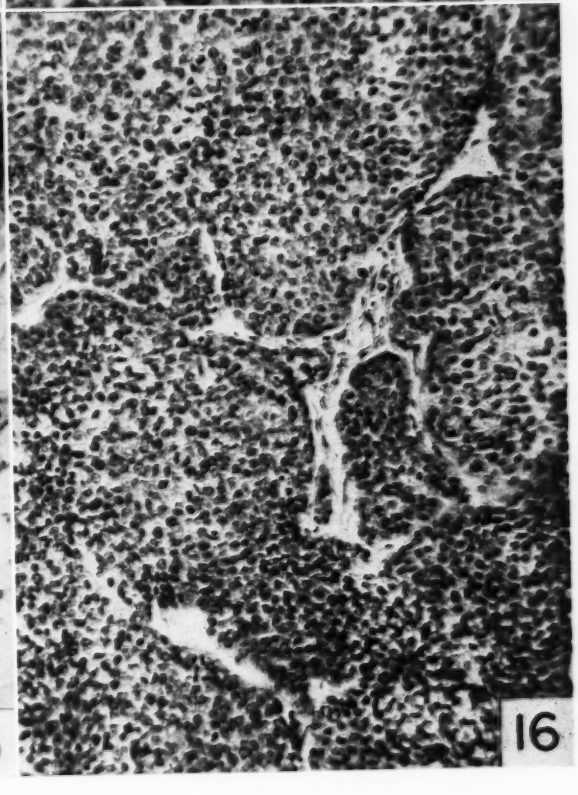

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Announcements

1953 Gordon Research Conferences, AAAS

Cancer Conference

(W. George Parks, Director; University of Rhode Island, Kingston, Rhode Island)

The Gordon Research Conferences for 1953, sponsored by the AAAS, will be held from June 16 to September 4 at Colby Junior College, New London, N.H. The facilities of the College have been made available for the Conferences.

The Gordon Research Conferences, AAAS, were established to stimulate research in universities, research foundations, and industrial laboratories. This purpose is achieved by an informal type of meeting consisting of the scheduled lectures and free discussion groups. Sufficient time is available to stimulate informal discussion among the members of a Conference. Meetings are held in the morning and in the evening, Monday through Friday, with the exception of Friday evening. The afternoons are available for recreation, reading, resting, or participation in discussion groups as the individual desires. This type of meeting is a valuable means of disseminating information and ideas which otherwise would not be realized through the normal channels of publication and scientific meetings. In addition, scientists in related fields become acquainted, and valuable associations are formed which result in collaboration and cooperative effort between different laboratories and in an informal exchange of ideas between persons actively interested in the subjects under discussion. The purpose of the program is not to review the known fields of chemistry, but primarily to bring experts up to date concerning the latest developments and their significance, and to provoke suggestions as to underlying theories and profitable approaches. To protect individual rights and to promote discussion, it is an established rule of each Conference that all information presented is not to be used without specific authorization of the individual concerned whether in formal presentation or in discussion. No publications are prepared as emanating from the Conferences.

The meetings will be held mornings, from 9 to 12, Monday through Friday, E.D.S.T. A second session will be held in the evening from 7:30 to 10:00 P.M., Monday through Thursday. There are no Friday evening meetings. Conference members are expected to release their rooms not later than Sunday morning unless they have made a reservation for the next Conference.

Accommodations are available for a limited number of women to attend each Conference, including wives who wish to accompany their husbands. All such requests should be made at the time of the request for attendance, because these limited accommodations will be assigned in the order that specific requests are received. Children 12 years and older can be accommodated at Colby Junior College. Rooms are available at several inns and hotels in the area if reservations are made in advance. No dogs or other animals will be permitted in the College dormitories.

Applications should be sent to the Director on or before May 1, 1953. Each applicant must state the institution or company with which he is connected and the type of work in which he is most interested. Attendance at each Conference is limited to 100. Requests for attendance at the Conferences, or for any additional information, should be addressed to W. George Parks, Director, Dept. of Chemistry, University of Rhode Island, Kingston, R.I. From June 15 to September 1, 1953, mail should be addressed to Colby Junior College, New London, N.H.

PROGRAM FOR THE 1953 CANCER CONFERENCE

Alfred Gellhorn, *Chairman*
Albert Tannenbaum, *Vice Chairman*

Aug. 31:

J. H. Quastel and A. Cantero. The Effect of Glucosamine on Tumor Growth.

Harris Busch. Studies of Oxidative Metabolism of Tumors *in Vivo*.

Jacob Furth. Character of I^{131}-induced Pituitary Growth; Hormonal and Histological Changes Accompanying Acquisition of Autonomy.

Robert Briggs and Thomas King. Transplantation of Embryonic Nuclei and the Problem of Nuclear Differentiation.

Sept. 1:

Frances L. Haven. Lipid Metabolism in Tumor-bearing Animals.

Robert Olson. Lipogenesis and Lipoprotein in Tumors.

C. Chester Stock, H. C. Reilly, Frederick S. Philips, Sonja Buckley, Donald Clarke, John Ehrlich and Quentin Bartz. An Anti-Tumor Preparation from a Soil Micro-organism. A Laboratory Investigation.

David A. Karnofsky. B. Clinical Trials.

Sept. 2:

Bjorne Pearson. Cytochemical Studies during Carcinogenesis.

M. J. Kopac. Submicro Enzyme Chemistry of Normal and Neoplastic Cells.

Hattie E. Alexander. A. The Biologic Activity of Desoxyribose Nucleic Acids as Hereditary Determinants in Bacteria.

Stephen Zamenhof. B. Recent Studies on the Chemistry of Heredity Determination in Bacteria.

Sept. 3:

Edwin E. Osgood. The Physiology of Normal and Leukemic Leukocytes. Other subjects and speakers to be announced.

Sept. 4:

Ernest L. Wynder. Clinical and Experimental Studies on the Etiology of Lung Cancer.

Johannes Clemmesen. Cancer Endemiology.

Southwestern Section

American Association for Cancer Research

The Southwestern Section of the American Association for Cancer research has 110 members at the present time. Eighty-nine of these are associate members, and 21 are full members. Programs of the Section were held immediately preceding the meetings of the Society for Experimental Biology and Medicine in Dallas on March 14, 1952, and in San Antonio November 7, 1952. The meeting in San Antonio was sponsored jointly by the Southwest Foundation for Research and Education and Trinity University. There were 30 members in attendance at the meeting in Dallas and 36 at the meeting in San Antonio. Guest lecturer at the spring meeting was J. B. Trunnell of the M. D. Anderson Hospital who spoke on "The Role of the Cancer Hospital in Cancer Research." Dr. Jacob Furth of the Biology Division, Oak Ridge National Laboratory spoke on "Conditioned and Autonomous Neoplasms of the Pituitary" at the fall meeting. The next meeting will be held in Oklahoma City on May 8, 1953. By-laws, modeled after those of the parent organization are being utilized to conduct the business of the society. Officers for the present year are Dr. C. L. Spurr, President, Dr. M. W. Shetlar, Vice-President, and Dr. W. J. Burdette, Secretary-Treasurer.

PROGRAM

Dallas, Texas
March 14, 1952

Dr. Walter Joel. Determination of Polysaccharides in Neoplasms by Histochemical Methods. *University of Oklahoma School of Medicine, Oklahoma City, Okla.*

Dr. Bela Halpert. Anlage Tumors about the Mouth. *Veterans Administration Hospital, Houston, Texas.*

Dr. Dudley Jackson. Cytological Study of Breast Secretions. *San Antonio, Texas.*

Dr. M. R. Shetlar. Investigations of the Anthrone Method for the Determination of Serum Polysaccharides. *Oklahoma Medical Research Foundation, Oklahoma City, Okla.*

Dr. Gilbert H. Fletcher. Dosimetry in Carcinoma of the Cervix. *M. D. Anderson Hospital, Houston, Texas.*

Dr. Joseph W. Kelso. Comparative Treatment of Carcinoma of the Cervix. *Oklahoma City, Okla.*

Drs. R. G. Gottschalk and A. Grollman. The Action of ACTH and Cortisone on Two Transplanted Tumors of Mice. *Veterans Administration Hospital, Houston, Texas.*

Dr. R. H. Rigdon. Experimental Cancer in White Pekin Ducks. *University of Texas Medical Branch, Galveston, Texas.*

Drs. Allen F. Reid, Margaret C. Robbins, Robert G. Budd, and Ellsie B. Ryan. Effects of Serum from Carcinomatous Mice on Erythrocyte Metabolism. *Southwestern Medical School of the University of Texas, Dallas, Texas.*

Dr. George W. Hagy (Introduced by Dr. C. P. Oliver). Preliminary Report of a Pilot Genetic and Clinical Study of Gastric Cancer. *The University of Texas. Austin, Texas.*

Drs. F. M. Lingenfelter and J. P. Dewar. Cylindroma of the Scalp. *Oklahoma City, Okla.*

Drs. J. M. Thuringer and A. A. Katzberg. (Presentation by Dr. A. A. Katzberg.) The Influence of Age on the Rates of Regeneration and Desquamation in the Human Epidermis. *University of Oklahoma Medical School, Oklahoma City, Okla.*

Dr. J. B. Trunnell (guest lecture). The Role of the Cancer Hospital in Research. *Section of Experimental Medicine, M. D. Anderson Hospital, Houston, Texas.*

PROGRAM

San Antonio, Texas
November 7, 1952

Drs. Morris Pollard and Robert Bussell. Role of the Spleen in Resistance to Experimental Tumors. *University of Texas Medical Branch, Galveston, Texas.*

Drs. B. Jirgensons and Jorge Awapara. Physicalchemical Studies on the Bence-Jones Protein from Multiple Myeloma. *M. D. Anderson Hospital, Houston, Texas.*

Drs. J. B. Loefer, R. B. Mefferd, Jr., and R. M. Nettleton, Jr. Variation in Host Susceptibility to Tumor Implants and Its Alteration. *Southwest Foundation for Research and Education and Trinity University, San Antonio, Texas.*

Dr. Iben Browning. Studies in the Normal Reproduction of the Protozoan, *Tetrahymena Geleii*. *American Optical Company, Buffalo, New York.*

Drs. C. M. Pomerat, D. Jackson, and W. C. McCormick. Activities of Cells in Fluid from the Breasts of Dogs. (Movie.) *University of Texas Medical Branch, Galveston, Texas, and San Antonio, Texas.*

Drs. C. P. Oliver and D. Jackson, Sr. Mother-Daughter Pairs in Breast Cancer Cases. *University of Texas, Austin, Texas and San Antonio, Texas.*

Drs. R. M. Nettleton, Jr., R. B. Mefferd, Jr. and J. B. Loefer. Some Features of the Metabolic Patterns of Susceptible and Resistant Hosts. *Southwest Foundation for Research and Education and Trinity University, San Antonio, Texas.*

Dr. Edward Krementz. Heterologous Transplantation of Neural Tumors. *Tulane University, New Orleans, Louisiana.*

Dr. Berne L. Newton. Studies on the Transplantability of Frozen Brown-Pearce Carcinoma. *Baylor Medical College, Houston, Texas.*

Dr. T. S. Painter. Royal Jelly Gland of the Bee. *The University of Texas, Austin, Texas.*

Drs. R. G. Gottschalk and H. C. Allen, Jr. Distribution of Radioactive Sulfur in Tissues of Patients with Chondrosarcoma. *Veterans Administration Hospital, Houston, Texas.*

Drs. M. R. Shetlar, William H. Knobloch, Mark Everett, and Harold Muchmore. Use of the Serum Polysaccharide Level as a Screening Test for Cancer. *University of Oklahoma, Oklahoma City, Oklahoma.*

Dr. Jacob Furth (guest lecture). Conditioned and Autonomous Neoplasms of the Pituitary. *Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee.*